Common and cryptic *Aspergillus* species – one health pathogens

Jessica Jane Talbot

Bachelor of Science (Vet) (Hons) Bachelor of Veterinary Science (Hons)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Veterinary Science)

Faculty of Science

University of Sydney

2018

Declaration

I declare that this thesis is the result of my own work and has not been submitted for another degree or qualification. All information derived from published or unpublished work of others has been acknowledged in the text, and a list of references is given for each publication and at the end of each chapter.

Jessica J. Talbot

June 2018

Acknowledgements

Firstly I wish to thank my husband Mark for his encouragement of me to pursue a PhD and support that has made it possible for me to undertake this task; without your love I would not have achieved this; thank you. Secondly, to Teddy, I hope this one day inspires you to be inquisitive of the world around you, in whatever capacity you choose. I love you both. Thirdly, to all my family and friends who have helped me through this challenging research period, for providing the love and the laughter; and to my cats, Gary, Vincent, Penny and Dan, whose great company and endless request for pats and food have maintained me.

I wish to thank all my supervisors Prof Vanessa Barrs, Prof Julia Beatty and Dr Sarah Kidd for their guidance throughout my PhD and assistance acquiring funds for this project. Thank you for helping me achieve this goal.

Thank you to all the owners of cats who had succumbed to infection for letting me into their homes to collect samples and re-telling their difficult and sad experience with feline aspergillosis. I hope this work provides some comfort that we are working towards understanding more about the fungi responsible for this disease, so we can uncover better treatment options for cats with this devastating infection.

My research into fungi started as an undergraduate Veterinary Science student as part of a research project with Prof Barrs who at the time was undergoing her own PhD research into feline sino-nasal aspergillosis. A keen interest in fungi affecting animals and humans quickly developed, and naturally progressed into my own PhD. From my early days, the mycological community has been extremely helpful, friendly and keen on collaboration, and extremely willing to impart their expertise and teach me their skills. A huge thank you to Dr Sarah Kidd (SA Pathology) and Dr Catriona Halliday (Westmead CIMID) for so willingly taking me on in their laboratories, making me feel welcome and teaching me about antifungal susceptibility testing and molecular identification of fungi respectively. A massive thank you to Dr Patricia Martin (Veterinary Pathology Diagnostic Services) for teaching me her techniques and tips for veterinary mycology – these were invaluable lessons that have served me well!

I have had incredible opportunities to travel far with this project, throughout Australia and also abroad to the Netherlands and Denmark on an Endeavour Research Fellowship. During this

time I was able to make many new colleagues and friends, and had invaluable experiences that have contributed to my professional growth. A huge thank you to my colleagues Dr Jos Houbraken, Dr Rob Samson (Westerdijk Institute) and Dr Jens Frisvad (Technical University Denmark) for taking on roles as my supervisors while I was abroad, and for teaching me many new skills in the field of Mycology. Thank you also to Dr Rob Samson for sharing his office with me and for all the team in the DTO department at Westerdijk Institute for including me in their lab and making me feel welcome.

A huge thank you to Mr Paul Thompson (Taronga Zoo Wildlife Hospital) for so diligently collecting and sending fungal cultures from affected birds in their collection, and to Dr Larry Vogelnest (Taronga Zoo Wildlife Hospital) for being a mentor of mine since my undergraduate days.

I would like to thank all the people who have contributed to my post-graduate experience. To my office colleagues (a special thanks to Anthea Brennan, Rebecca Gooley, Alicia McLuckie and Joanna Whitney for their friendship, fun times and shared angst), fellow lab workers, clinicians and field workers who have assisted me throughout this time, and the many animals I have met along the way; dogs, cats, birds and even Tasmanian Devils.

I hope you enjoy reading this thesis – fungi are phenomenally resilient and adaptable life forms that have the ability to survive under extreme conditions. They can be admired, but not adored, given their ability to use these traits to cause disease in people and animals, and their ability to wage war against our best medical treatments available.

Table of Contents

Authorship attribution statement

Included in this thesis are manuscripts that I have published during my candidature in peer-reviewed journals. I am the primary author of four publications. I am co-primary author for one publication. In accordance with the University of Sydney policy on submission of a thesis by publication, these manuscripts form the research chapters of this thesis. A list of the manuscripts included in this thesis is found below, followed by the co-authorship confirmation statements for each published work (signed copies are held on file, but not included in this document for privacy reasons). Additionally, each chapter includes the relevant supplementary material.

Following this is listed an unpublished manuscript that I have included as the final research chapter for my thesis. I am the primary author of this manuscript, and have also included co-authorship confirmation statements for this unpublished work.

Following on from this is a list of additional publications I contributed to throughout my candidature which are not included as primary research chapters but are examples of additional fungal related work completed during this time. These manuscripts are located in the appendix, along with contribution information.

Permission to include these publications has been sought from the Publisher and corresponding authors.

Published manuscripts included in this thesis

Part of Chapter 2 of this thesis is published as:

Talbot J. J., Barrs V.R. One-health pathogens in the *Aspergillus viridinutans* complex. *Medical Mycology.* 2017; doi: 10.1093/mmy/myx016.

Part of Chapter 3 of this thesis is published as:

Talbot J. J., Houbraken J., Frisvad J. C., Samson R. A., Kidd S. E., Pitt J., Lindsay S., Beatty J. A., Barrs V. R. Discovery of *Aspergillus frankstonensis* sp. nov. during environmental

sampling for animal and human fungal pathogens. *PLOS ONE*. 2017; 12 (8): e0181660. https://doi.org/10.1371/journal.pone.0181660.

Part of Chapter 4 of this thesis is published as:

Talbot J. J., Kidd S. E., Martin P., Beatty J. A., Barrs V. R. Azole resistance in canine and feline isolates of *Aspergillus fumigatus. Comparative Immunology, Microbiology and Infectious Diseases.* 2015; 42: 37-41.

Part of Chapter 5 of this thesis is published as:

Talbot J. J., Thompson P., Vogelnest L., Barrs V. R. Identification of pathogenic *Aspergillus* isolates from captive birds in Australia. *Medical Mycology*. 2017; doi: 10.1093/mmy/myx137.

Part of Chapter 6 of this thesis is published as:

Talbot J. J., Subedi S., Halliday C. L., Hibbs D. E., Lai F., Lopez-Ruiz F. J., Harper L., Park R., Cuddy W., Biswas C., Cooley L., Carter D., Sorrell T. C., Barrs V. R., Chen S. C-A. Surveillance for azole resistance in clinical and environmental isolates of *Aspergillus fumigatus* in Australia and *cyp51A* homology modelling of azole-resistant isolates. *Journal of Antimicrobial Chemotherapy*. 2018; doi: 10.1093/jac/dky187.

Unpublished manuscript included in this thesis

Part of Chapter 7 of this thesis is an unpublished manuscript:

Talbot J. J., Samson R. A., Kidd S. E., Meis J. F., Hagen F, Hibbs D. E., Lai F., Verweij P. E., Barrs, V. R., Houbraken J. *cyp51A* mutations in Clinical and Environmental Isolates of the *Aspergillus viridinutans* Species Complex. Unpublished manuscript, June 2018.

Additional manuscripts included within the appendix

Taylor A., **Talbot J.J.,** Bennett P., Martin P., Makara M., Barrs V.R. Disseminated *Scedosporium prolificans* infection in a Labrador retriever with immune mediated haemolytic anaemia. *Medical Mycology Case Reports.* 2014; 6:66-69.

Barrs V.R., Urjvari B., Peeters I., Dhand N., Beatty J.A., Johnson L., **Talbot J.,** Billen F., Martin P., Madsen T., Belov K. Detection of *Aspergillus*-specific antibodies by agar gel immunodiffusion and IgG ELISA for diagnosis of feline upper respiratory tract aspergillosis. *The Veterinary Journal*. 2015; 203:285-289.

Duchaussoy A.-C., Rose A., **Talbot J.J.,** Barrs V.R. Gastrointestinal granuloma due to *Candida albicans* in an immunocompetent cat. *Medical Mycology Case Reports*. 2015; 10:14-17.

Tamborini A., Robertson E., **Talbot J.J.,** Barrs V.R. Sinonasal aspergillosis in a British shorthair cat in the United Kingdom. *Journal of Feline Medicine and Surgery Open Reports*. 2016; 2(1):1-6.

Confirmation of co-authorship of published work

Publication

Talbot JJ, Barrs VR. One-health pathogens in the *Aspergillus viridinutans* complex. *Medical Mycology*. 2017; doi: 10.1093/mmy/myx016.

Authors' contributions

Jessica J. Talbot, the candidate, performed the literature review and wrote this research article. Vanessa R. Barrs assisted in drafting and finalising the manuscript.

I, as a co-author, endorse that the level of contribution by myself and the candidate indicated above is appropriate.

Confirmation of co-authorship of published work

Publication

Talbot JJ, Houbraken J, Frisvad JC, Samson RA, Kidd SE, Pitt J, Lindsay S, Beatty JA, Barrs VR. Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for animal and human fungal pathogens. *PLOS ONE*. 2017; 12 (8): e0181660. https://doi.org/10.1371/journal.pone.0181660

This study was designed by Jessica J. Talbot, the candidate and Prof Vanessa R. Barrs. Prof Julia A. Beatty, Dr Sarah E. Kidd, Dr John Pitt, Dr Jos Houbraken and Prof Robert A. Samson provided guidance in the study design. Jessica J. Talbot collected all environmental samples and performed all laboratory work (fungal isolation from environmental samples, fungal DNA extraction and PCR, sequence analysis, fungal growth and mating studies, fungal metabolite extraction), with the exception of electron microscopy images which were taken by Dr Sue Lindsay, and processing of metabolite extracts through a High-Performance Liquid Chromatography (HPLC) machine which was performed by Prof Jens C. Frisvad. Prof Jens C. Frisvad also provided analysis of extrolite metabolite profiles. Phylogenetic tree analysis was performed by Jessica J. Talbot and Dr Jos Houbraken. Jessica J. Talbot wrote this research article. Dr Jos Houbraken, Prof Jens C. Frisvad, Dr Sarah E. Kidd, Dr John Pitt, Prof Julia A. Beatty and Prof Vanessa R. Barrs assisted in drafting and finalising the manuscript.

I, as a co-author, endorse that the level of contribution by myself and the candidate indicated above is appropriate.

Confirmation of co-authorship of published work

Publication

Talbot J. J., Thompson P., Vogelnest L., Barrs V. R. Identification of pathogenic *Aspergillus* isolates from captive birds in Australia. *Medical Mycology.* 2017; doi: 10.1093/mmy/myx137.

Authors' contributions

Jessica Talbot, the candidate, performed all experimental work (fungal DNA extraction and PCR), analysis of post-mortem records and wrote this research article. Jessica Talbot and Prof Vanessa Barrs designed the study and Mr Paul Thompson, Dr Larry Vogelnest provided guidance in the study design and assisted in drafting and finalising the manuscript.

I, as a co-author, endorse that the level of contribution by myself and the candidate indicated above is appropriate.

Confirmation of co-authorship of published work

Publication

Talbot J. J., Kidd S. E., Martin P., Beatty J. A., Barrs V. R. Azole resistance in canine and feline isolates of *Aspergillus fumigatus. Comparative Immunology, Microbiology and Infectious Diseases.* 2015; 42: 37-41.

Authors' contributions

Jessica Talbot, the candidate, performed all experimental work (antifungal susceptibility testing and *cyp51A* gene sequencing), and wrote this research article. Jessica Talbot designed this study and Prof Vanessa **R.** Barrs and Dr Sarah E. Kidd provided guidance in the study design. Dr Sarah E. Kidd, Dr Patricia Martin, Prof Julia A. Beatty and Prof Vanessa R Barrs assisted in drafting and finalising the manuscript.

I, as a co-author, endorse that the level of contribution by myself and the candidate indicated above is appropriate.

Confirmation of co-authorship of published work

Publication

Talbot J.J., Subedi S., Halliday C. L., Hibbs D. E., Lai F., Lopez-Ruiz F. J., Harper L., Park R. F., Cuddy W. S., Biswas C., Cooley L., Carter D., Sorrell T. C., Barrs V. R., Chen S. C-A. Surveillance for azole resistance in clinical and environmental isolates of *Aspergillus fumigatus* in Australia and *cyp51A* homology modelling of azole-resistant isolates. *Journal of Antimicrobial Chemotherapy*. 2018; doi: 10.1093/jac/dky187.

Authors' contributions

Jessica J. Talbot, the candidate, developed this study design in collaboration with Prof Vanessa R. Barrs, A/Prof Sharon C-A. Chen, Dr Catriona Halliday and Dr Shradha Subedi. Jessica J. Talbot and Dr Shradha Subedi are listed as equal contributors for this publication having contributed equally to experimental work (Dr Shradha Subedi performed fungal DNA extraction and PCR identification on human isolates and Jessica J. Talbot did this for environmental and animal isolates). Jessica J. Talbot collected soil samples from properties in NSW and performed isolation of *Aspergillus* species from soil. Prof Vanessa R. Barrs provided assistance with soil isolation. Additional soil samples were provided by Dr William S. Cuddy, Dr Fran J. Lopez-Ruiz, Dr Lincoln Harper and Dr Robert F. Park. Additional human origin *A. fumigatus* isolates were provided by Dr Louise Cooley. Jessica J. Talbot, Prof Vanessa R. Barrs, Dr Shradha Subedi and Dr Catriona Halliday performed azole susceptibility screening testing (VIPcheck™). Dr Catriona Halliday performed antifungal susceptibility testing of human, environmental and animal isolates (Sensititre™). Dr Chayanika Biswas performed *cyp51A* analysis on human isolates, and Jessica J. Talbot performed this on environmental isolates. Prof David E. Hibbs and Ms Felcia Lai performed the protein homology modelling and analysis. Jessica J. Talbot wrote this research article and all authors assisted in drafting and finalising the manuscript.

I, as a co-author, endorse that the level of contribution by myself and the candidate indicated above is appropriate.

Confirmation of co-authorship of unpublished work

Unpublished manuscript

Talbot J. J., Frisvad, J.C., Meis J. F., Hagen F, Verweij P. E., Hibbs D. E., Lai F., Samson R. A., Kidd S. E., Barrs, V. R., Houbraken J. *Cyp51A* mutations in Clinical and Environmental Isolates of the *Aspergillus viridinutans* Species Complex.

Authors' contributions

Jessica J. Talbot, the candidate, designed this study with guidance from Dr Jos Houbraken, Prof Vanessa R. Barrs, Prof Robert A. Samson and Prof Paul E. Verweij. Jessica J. Talbot performed all experimental work (Sensititre™ antifungal susceptibility testing, *cyp51A* gene sequencing and analysis, fungal metabolite extraction), with the exception of CLSI antifungal susceptibility testing which was arranged by Dr Jacques Meis and Dr Ferry Hagen, *cyp51A* homology modelling and analysis which was performed by Prof David E. Hibbs and Ms Felcia Lai, and processing of metabolite extracts through a high powered liquid chromatography (HPLC) machine which was performed by Prof Jens C. Frisvad. Prof Jens C. Frisvad also provided analysis of extrolite metabolite profiles. Phylogenetic tree analysis was performed by Jessica J. Talbot and Dr Jos Houbraken. Jessica J. Talbot wrote this research article and all authors assisted in drafting and finalising the manuscript.

I, as a co-author, endorse that the level of contribution by myself and the candidate indicated above is appropriate.

Presentations

University of Sydney Faculty of Veterinary Science Post-graduate Conference: Early stage candidature seminar presentation. Sydney, Australia, Jul 2014.

International Feline Retrovirus Research Symposium & International Society for Companion Animal Infectious Disease Conference: Abstract presentation. Niagara-on-the-lake, Canada, October 2014.

Marie Bashir Institute Colloquium Sydney: Poster presentation. Sydney, Australia, Nov 2014.

International Society for Human and Animal Mycoses Conference: Abstract presentation. Melbourne, May 2015.

Westerdijk Institute Seminar Series: Invited research presentation. Utrecht, the Netherlands, Jan 2016.

International Feline Retrovirus Research Symposium & International Society for Companion Animal Infectious Disease Conference: Abstract presentation. Bristol, United Kingdom, October 2016.

University of Sydney Faculty of Science Sydney School of Veterinary Science Post-graduate Conference: Late stage candidature seminar presentation. Sydney, Australia, Oct 2017.

Awards

Australian Government Endeavour Research Fellowship (2015-2016).

Faculty of Science, Sydney School of Veterinary Science Post-graduate conference: Late candidature best presentation award (2017).

Abbreviations

ABPA	allergic bronchopulmonary aspergillosis
ARAF	azole-resistant *Aspergillus fumigatus*
AVSC	*Aspergillus viridinutans* species complex
BAL	bronchoalveolar lavage
BenA	beat-tubulin
CalM	calmodulin
CF	cystic fibrosis
CLSI	Clinical Laboratory Standards Institute
CPA	chronic pulmonary aspergillosis
CT	computed tomography
DMI	demethylation inhibitors
DNA	deoxyribose nucleic acid
ECV	epidemiological cut-off value
EUCAST	European Committee for Antibiotic Susceptibility Testing
IA	invasive aspergillosis
ICU	intensive care unit
IgG	immunoglobulin G
IPA	invasive pulmonary aspergillosis
ITS	internal transcribed spacer
MIC	minimum inhibitory concentration
MCM7	minichromosome maintenance factor 7
MEC	minimum effective concentration
MRI	magnetic resonance imaging

RPB2	RNA polymerase II genes
SNA	sino-nasal aspergillosis
SOA	sino-orbital aspergillosis
TR	tandem repeat
URTA	upper respiratory tract aspergillosis

Abstract

Fungal *Aspergillus* species cause invasive and chronic disease in humans and other animals, mostly due to the ubiquitous *Aspergillus fumigatus*, and triazole therapeutics are the first line antifungal defence. However, in the past decade there has been a global increase in disease due to "cryptic" *Aspergillus* species, and azole resistance development amongst *A. fumigatus* and cryptic *Aspergillus* species has complicated treatment. In particular, fungal infections due to *Aspergillus viridinutans* species complex (AVSC) members are being increasingly reported in human and veterinary medicine. Invasive upper respiratory tract infection due to *A. felis* in cats has been the most commonly reported syndrome. Although the first member of the complex, *A. viridinutans* sensu stricto was discovered in Australia in 1954, the ecological niche and prevalence of other *Aspergillus viridinutans* species complex members in Australia, including *A. felis*, has not been investigated. Additionally, the most common *Aspergillus* species causing avian aspergillosis, commonly affected animal species, in Australia is unknown. The prevalence of and risk of exposure to azole resistant *A. fumigatus* (ARAF) due to *cyp51A* mutations in clinical and environmental isolates in Australia is also largely unknown. This thesis investigates cryptic and common *Aspergillus* species belonging to *A.* section *Fumigati*; their prevalence in Australia and virulence factors affecting the efficacy of the most commonly used antifungal therapeutics, triazole therapeutics.

An environmental investigation for cryptic fungal species, with a focus on AVSC members in the Australian environment is documented, and the discovery of a new fungal species within the AVSC is described. Soil and air (Merck Millipore MAS-100 air sampler) samples were collected from yards and gardens of 8 Australian homes where cats diagnosed with aspergillosis due to *Aspergillus viridinutans* species complex members lived (NSW 5, Vic 2, ACT 1), and from the Mornington Peninsula region in VIC, where *A. viridinutans* sensu stricto was originally isolated in the 1950's. Soil samples were diluted, heat treated, and soil and air samples were cultured on specialised agar for up to 3 weeks at 37 ^{o}C. Cultures with morphological features consistent with *Aspergillus* species underwent DNA extraction, PCR and sequencing of the internal transcribed spacer (ITS) region and partial beta tubulin gene (BenA) for species identification. Isolates belonging to the AVSC then underwent further characterisation including growth on multiple agar types and at varying temperatures, PCR and sequencing of the calmodulin (CalM), minichromosome maintenance factor 7 (MCM7), actin

and RNA polymerase II genes (RPB2) as well as extrolite extraction and analysis. Of 104 isolates recovered, 61% were *A. fumigatus*, 9% were AVSC members and 30% were other *Aspergillus* species, including 6 known human and animal pathogenic species (*A. fischeri, A. thermomutatus, A. lentulus, A. laciniosus, A. fumisynnematus* and *A. hiratsukae*). AVSC members were not isolated from the home environments of cats; however *A. felis*-clade isolates were recovered from the Mornington Peninsula (Frankston, Victoria) where a new fungal species belonging to the complex was also isolated, named *A. frankstonensis.* Although the prevalence of other cryptic and pathogenic *Aspergillus* species was common, the risk of direct environmental exposure to AVSC members in areas where humans and cats co-habitate in Australia is low, and they appear to have a particular environmental niche in Frankston, Victoria.

The most common cause of avian aspergillosis amongst captive birds in Australia is also described. Thirty fungal isolates from 30 birds with confirmed avian aspergillosis were received from zoological parks (NSW 23, SA 3), poultry farms (NSW 3) and private practice (Qld 1). All cultures had morphological features consistent with *Aspergillus* species and underwent DNA extraction, PCR and sequencing of the ITS region and partial BenA gene for identification to species level. *A. fumigatus* is identified as the most common cause of avian aspergillosis in captive birds in Australia (87%). *A. restrictus* is also identified here for the first time as a cause of avian aspergillosis. *A. flavus* and *A. nidulans* were also agents of disease. Cryptic *Aspergillus* species were not found to cause disease in captive birds in Australia.

The prevalence of azole resistance amongst *A. fumigatus* isolates from clinical (human and veterinary) and environmental (agricultural and domestic) samples in Australia is documented over two studies. The first study investigated antifungal susceptibility profiles in veterinary isolates of *A. fumigatus* from dogs (n=46) and cats (n=4) with fungal rhino-sinusitis in Australia (n=33), the USA (n=10) and Belgium (n=7). Itraconazole resistance was detected in one Australian isolate sampled from a dog in the early 1990's. *Cyp51A* sequencing of this isolate revealed the F46Y single point mutation; however this mutation has not been confirmed to confer azole resistance. There was a low prevalence of ARAF in Australian, USA and Belgium veterinary isolates, and no evidence of emerging azole resistant *A. fumigatus* in veterinary isolates. The second study investigates azole resistance amongst both Australian clinical and environmental isolates. The identities of 354 *A. fumigatus* isolates (185 environmental [64 azole exposed, 121 azole naïve], 21 veterinary [14 avian, 5 canine, 2 feline] and 148 human clinical

isolates) were confirmed by sequencing of the partial BenA gene and isolates screened for azole susceptibility using a commercial test kit (VIPcheck™). Antifungal susceptibility was tested for any isolates demonstrating resistance (n=8) as well as non-resistant controls (n=192) using a commercially available microdilution assay (Sensititre™ YeastOne™ YO10), which confirmed azole resistance in three isolates of human origin. *Cyp51A* sequencing of resistant isolates revealed the *cyp51A* tandem repeat (TR) and single point mutation TR_{34}/L98H in one multi-azole resistant isolate, and two itraconazole and posaconazole resistant isolates harbouring the G54R single point mutation. The prevalence of ARAF in Australia was low, detected in only 0.85% of all isolates tested (3/354), and was associated with mutations in the *cyp51A* gene.

Investigation of antifungal susceptibility testing and amplification of the *cyp51A* gene in cryptic AVSC members is described. A total of 56 AVSC isolates of both environmental (n=26), clinical (7 human and 19 veterinary) and unknown (n=4) origins were examined. DNA extraction and amplification of the *cyp51A* gene using novel degenerate primers, mircobroth dilution antifungal testing (CLSI reference method (n=37)) and *cyp51A* crystal structure protein modelling based on *Saccharomyces cerevisiae* were performed. CLSI testing detected very high minimum inhibitory concentrations (MICs) to azoles in 84% of isolates. Compared to the wild-type *A. fumigatus cyp51A* gene, high rates of *cyp51A* mutations were detected in the cohort. *Cyp51A* protein homology modelling of mutations unique to isolates with high minimum inhibitory concentrations to azole drugs tested (G138C/T215S; S197C/Q340R; A63S/L327P/V396A; A103T/V101L/A234V/I360V/V428I/G505R/Q423D/F478V) did not significantly change the overall protein structure to confer resistance.

This program of research has furthered our understanding of the epidemiology of common and cryptic pathogenic *Aspergillus* species causing disease in humans and other animals in Australia, including aspects of disease pathogenesis in cats, and causes of treatment failure, including mechanisms of azole resistance in *A. fumigatus* and cryptic species of the AVSC. Key findings include a moderate risk of exposure to known pathogenic and cryptic *Aspergillus* species, but a low risk of exposure to AVSC members in indoor and outdoor domestic environments in Australia; an environmental niche for AVSC members in Frankston, Australia; the discovery of a new cryptic *A.* sect. *Fumigati* species within the AVSC, *A. frankstonensis* present at least in Australia and has demonstrated high minimum inhibitory concentration values to triazole drugs; *A. fumigatus* as the most common cause of avian aspergillosis in

Australia with no cryptic species infections identified; *A. restrictus* as a pathogenic agent of avian aspergillosis; a low prevalence of ARAF amongst Australian isolates from clinical (human and veterinary) and environmental origins; *cyp51A* sequencing in cryptic AVSC members; and antifungal susceptibility profiles of AVSC members. This research represents an important step in our understanding of one-health *Aspergillus* species pathogens from clinical and environmental settings, namely the importance of screening for both cryptic and azole resistant *A.* sect. *Fumigati* species.

Chapter 1. Introduction

1.1 Introduction

Aspergillosis, an infection with *Aspergillus* fungal species, can present as a variety of disease forms from non-invasive localised to invasive focal or invasive disseminated infections. Antifungal azole drugs are first line therapeutics for this disease. In the past 20 years discoveries in the field of mycology have furthered our knowledge of pathogenic *Aspergillus* species and the efficacy of first line of defence azole therapeutics. These discoveries helped highlight some of the causes of treatment failure amongst aspergillosis patients.

Advances in molecular identification techniques and polyphasic taxonomy have led to the recognition of previously unknown and unrecognised *Aspergillus* species as opportunistic pathogens (Hong et al., 2005, Samson et al., 2007, Samson et al., 2014). This advancement has identified "cryptic" *Aspergillus* species, previously mistakenly identified as *Aspergillus fumigatus*, as a cause of disease in humans and other animal species. Correct identification of cryptic species is important as cryptic species infections are often associated with invasive disease unresponsive to common antifungal therapies, with a high mortality rate (Alhambra et al., 2008, Vinh et al., 2009a, Vinh et al., 2009b, Coelho et al., 2011, Barrs et al., 2013, Alastruey-Izquierdo et al., 2014), particularly cryptic species belonging to the *Aspergillus viridinutans* species complex (AVSC). These fungi cause invasive disease in humans (Balajee et al., 2006, Alcazar-Fuoli et al., 2008, Vinh et al., 2009a, Vinh et al., 2009b, Sugui et al., 2010, Posteraro et al., 2011, Coelho et al., 2011, Gyotoku et al., 2012, Andersen et al., 2012, Escribano et al., 2013, Barrs et al., 2013, Peláez et al., 2013, Alastruey-Izquierdo et al., 2013, Sugui et al., 2014, Nováková et al., 2014, Tamiya et al., 2015) and interestingly are the most commonly reported cause of invasive sino-orbital aspergillosis in cats, with most cases reported in Australia and due to the AVSC member *A. felis* (Barrs et al., 2012, Barrs et al., 2013). This disease in cats has a high mortality rate, with the largest study reporting a 92% mortality rate (Barrs et al., 2012). Although studies have documented the discovery of *A. felis* in the environment in several global locations (Barrs et al., 2013, Nováková et al., 2014, Gonçalves et al., 2016), its prevalence, and those of other AVSC and cryptic *Aspergillus* species in Australia, is largely unknown.

Cryptic species are not however the only threat to aspergillosis treatment and prognosis. Since first reported in 1997 (Denning DW et al., 1997), azole resistant *A. fumigatus* isolates have been increasingly identified amongst clinical and environmental isolates throughout the world. This is particularly concerning as antifungal therapeutic choices are limited, with azoles being one of the few approved oral antifungal treatments available for aspergillosis (Patterson et al., 2016). While azole resistant *A. fumigatus* has been reported in isolates from Australian human patients (Kidd et al., 2015), no large-scale surveillance studies have investigated clinical and environmental isolates of *A. fumigatus*.

My research began as a yearlong research project I completed as an undergraduate Bachelor of Veterinary Science student. Under the supervision of a Small Animal Medicine Specialist, Professor Vanessa Barrs, I began researching the most common cause of aspergillosis amongst dogs in Australia using molecular fungal identification techniques. Over the course of the year this project developed into a multicentre international collaboration, and my interest in veterinary research and fungal pathogens in animals and humans was only further enhanced. I was exposed to clinical examination and investigation of dogs and cats with aspergillosis performed at the University Veterinary Teaching Hospital Sydney and was able to assist with diagnosis through application of my newly acquired technical skills. My project determined that the most common cause of upper respiratory tract aspergillosis in dogs was *A. fumigatus*, and that the rate of cryptic species causing infection in dogs was interestingly much lower (2%) than that of cats, where 65% of upper respiratory tract aspergillosis infections involve the sino-orbital structures, and 100% of these reported sino-orbital infections are caused by cryptic *Aspergillus* species (Talbot et al., 2014, Barrs and Talbot, 2014). The marked differences in disease presentation I had seen between dogs and cats, differences in the aetiological agent of infection, and poor clinical outcomes experienced by both groups cemented my desire to continue research into the pathogenesis of this disease, focusing on the pathogen-environment interaction, and why antifungal therapies may not be curative.

The overall objective of my research was to determine the prevalence of common and cryptic *Aspergillus* one-health pathogens in Australia, the environmental niche of specific agents of aspergillosis in cats in Australia (particularly AVSC members) and antifungal susceptibility patterns in clinical and environmental *A.* sect. *Fumigati* isolates (particularly *A. fumigatus* and AVSC members). Specific aims were to investigate the environmental prevalence of cryptic

Aspergillus species in Australia (Chapter 3), aetiological agent/s of avian aspergillosis in captive birds in Australia (Chapter 4), the prevalence of azole resistant *A. fumigatus* amongst Australian environmental and clinical isolates (Chapters 5, 6) and to further examine azole susceptibility profiles and potential causes of high minimum inhibitory concentrations (MICs) to antifungal azoles in the cryptic AVSC members (Chapter 7). To achieve this, I collected environmental (Chapters 3 and 6) and clinical (Chapters 4 - 6) *Aspergillus* species isolates from multiple locations in Australia. I also spent four months at the Westerdijk Institute in the Netherlands where I accessed their collection of AVSC isolates, for further examination.

In Chapter 3, an environmental investigation for cryptic *Aspergillus* species in Australia is described. The specific aim of this study was to investigate the Australian environmental prevalence of cryptic *Aspergillus* species causing infection in cats (members of the AVSC), as most global reports of feline upper respiratory tract aspergillosis (URTA) were from Australia, and the risk of environmental exposure to animals including humans was unknown; so the investigation included sampling air and substrates from the homes of cats with confirmed URTA. Additionally, samples were collected from a nature reserve in Frankston, Victoria, Australia because this was where the founding member of this complex, *A. viridinutans*, was discovered in the 1950s. *Aspergillus* species isolated from samples were identified based on phenotypic and molecular methods. This revealed common and cryptic pathogenic *Aspergillus* species were common in domestic Australian environments, but interestingly AVSC members were only isolated from Frankston. To further characterise the AVSC isolates I found in the Australian environment I visited the Westerdijk Institute in Utrecht, the Netherlands, funded by an Endeavour Research Fellowship from the Australian Government. There further analysing the isolates using polyphasic taxonomy with Professor Robert Samson and Dr Jos Houbraken, I discovered a novel fungal species within the AVSC, *A. frankstonensis*.

This visit also allowed me to further examine all AVSC isolates in the Westerdijk culture collection in addition to my own (from Chapter 3) and to investigate potential defence mechanisms. Clinical investigations had shown that infections due to these species were often non-responsive to antifungal azole therapies and it was hypothesised this could be due to mutations in the *cyp51A* gene as has been increasingly detected in *A. fumigatus* species. This included amplification of the *cyp51A* gene (the target gene of azole drugs), and several novel *cyp51A* mutations discovered are described in Chapter 7. I also

visited Professor Jens Frisvad of the Technical University of Denmark to use high-performance liquid chromatography (HPLC) to analyse the secondary metabolite profiles of my new species (Chapter 3). Kindly, the Westerdijk Institute shared their AVSC isolates and upon my return to Australia I tested antifungal susceptibilities using a commercial microbroth dilution method commonly used in Australian Mycology Reference Laboratories (Sensititre™ YeastOne™ YO8, TREK Diagnostic Systems Ltd., OH, USA), while colleagues in Europe performed the Clinical Laboratory Standards Institute (CLSI) antifungal susceptibility testing method to compare results (Clinical Laboratory Standards Institute (CLSI), 2017). I also received an isolate from an Australian human patient in this time from Dr Sarah Kidd, SA Pathology, Adelaide, SA, Australia, which I identified as *A. felis* (a member of the AVSC), and so the isolate was added to the study. High MICs to azoles were detected in most isolates on CLSI testing, (84 %) but only two had *cyp51A* mutations previously associated with resistance in *A. fumigatus* species. This led to further collaboration at the University of Sydney with Professor David Hibbs and his PhD student Ms Felcia Lai into *cyp51A* protein modelling to determine the potential significance of these and other mutations only seen amongst isolates with high azole MIC values on CLSI testing. This study concluded that mutations in AVSC *cyp51A* did not significantly change the overall protein structure to confer resistance. This study also presents antifungal susceptibility testing results for AVSC against luliconazole, with low MIC values reported for this different class antifungal drug. Chapter 7 describes the findings of this investigation.

The prevalence of azole resistance in *A. fumigatus* was examined initially in a study focusing on isolates from canine and feline URTA. This study included Australian, North American (USA) and Belgian isolates and is detailed in Chapter 5. Multi- azole resistance was detected in an Australian canine isolate from the early 1990s; however *cyp51A* analysis only revealed one mutation, F46Y, which has been associated with both resistant and wild-type (susceptible) isolates of *A. fumigatus,* making its significance undetermined. The environmental cryptic species investigation (Chapter 3) had recovered many *A. fumigatus* isolates and, following the publication by Dr Sarah Kidd in 2015 that identified the presence of azole resistance amongst Australian human clinical isolates, the idea of screening for azole resistance amongst my environmental isolates developed. It progressed into a multi-centre collaboration with Infectious Disease Specialist Dr Sharon Chen's group at CIMID, Westmead Hospital, NSW, Australia for which I collected and

processed veterinary (including isolates from Chapter 4) and environmental (samples from agriculture, viticulture, hospital grounds, and a selection of isolates from domiciles and the nature reserve from Chapter 3) isolates of *A. fumigatus*, and Dr Chen's group screened human clinical isolates of *A. fumigatus.* The results of this study described in Chapter 6 revealed a low prevalence of azole resistance in Australia (2%) which was only detected in isolates from human patients. This provided the opportunity to collaborate with Professor Hibbs' group again to model the mutations found in human clinical isolates, detailed in Chapter 6.

Throughout my investigation I received prospective isolates from clinical cases of avian aspergillosis. Chapter 4 describes the findings of this investigation into avian aspergillosis amongst captive birds in Australia. This study revealed the most common cause of aspergillosis in Australian captive birds is *A. fumigatus* (87%), with no cryptic species identified amongst the isolates. *Aspergillus fumigatus* isolates from this study were also screened for azole resistance in Chapter 6. This study also reports for the first time *A. restrictus* as a fungal pathogen in birds.

Chapter 8 includes a discussion of the research findings from Chapters 3 - 7 and recommendations for future research on aspergillosis in humans and other animals, highlighting the need for continued surveillance for both cryptic species and azole resistance amongst clinical and environmental *Aspergillus* isolates.

1.2 References

ALASTRUEY-IZQUIERDO, A., ALCAZAR-FUOLI, L. & CUENCA-ESTRELLA, M. 2014. Antifungal susceptibility profile of cryptic species of *Aspergillus*. *Mycopathologia*, 178**,** 427-433.

ALASTRUEY-IZQUIERDO, A., MELLADO, E., PELÁEZ, T., PEMÁN, J., ZAPICO, S., ALVAREZ, M., RODRÍGUEZ-TUDELA, J. L., CUENCA-ESTRELLA, M. & AND FILPOP STUDY GROUP 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrobial Agents and Chemotherapy,* 57**,** 3380-3387.

ALCAZAR-FUOLI, L., MELLADO, E., ALASTRUEY-IZQUIERDO, A., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2008. *Aspergillus* Section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. *Antimicrobial Agents and Chemotherapy,* 52**,** 1244-1251.

ALHAMBRA, A., CATALÁN, M., MORAGUES, M. D., BRENA, S., PONTÓN, J., MONTEJO, J. C. & DEL PALACIO, A. 2008. Isolation of *Aspergillus lentulus* in Spain from a critically ill patient with chronic obstructive pulmonary disease. *Revista iberoamericana de micología,* 25**,** 246-249.

ANDERSEN, C. T., BECHENSTEEN, A. G., HESSTVEDT, L. & GAUSTAD, P. 2012. Multiple brain abscesses caused by *Aspergillus viridinutans* in a leukemic child. *Mycoses,* 55**,** 75.

BALAJEE, S. A., NICKLE, D., VARGA, J. & MARR, K. A. 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryotic Cell,* 5**,** 1705-1712.

BARRS, V. R., HALLIDAY, C., MARTIN, P., WILSON, B., KROCKENBERGER, M., GUNEW, M., BENNETT, S., KOEHLMEYER, E., THOMPSON, A., FLIEGNER, R., HOCKING, A., SLEIMAN, S., O'BRIEN, C. & BEATTY, J. A. 2012. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *The Veterinary Journal,* 191**,** 58-64.

BARRS, V. R. & TALBOT, J. J. 2014. Feline aspergillosis. *Veterinary Clinics of North America: Small Animal Practice,* 44**,** 51-73.

BARRS, V. R., VAN DOORN, T. M., HOUBRAKEN, J., KIDD, S. E., MARTIN, P., PINHEIRO, M. D., RICHARDSON, M., VARGA, J. & SAMSON, R. A. 2013. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One,* 8**,** e64871.

CLINICAL LABOARTORY STANDARDS INSTITUTE (CLSI) 2017. M38, 3rd edition *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi.* Clinical and Laboratory Standards Institute: CLSI, Wayne, PA.

COELHO, D., SILVA, S., VALE-SILVA, L., GOMES, H., PINTO, E., SARMENTO, A. & PINHEIRO, M. D. 2011. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. *Medical Mycology,* 49**,** 755-759.

DENNING DW, VENKATESWARLU K, OAKLEY KL, ANDERSON MJ, MANNING NJ, STEVENS DA, WARNOCK DW & KELLY SL 1997. Itraconazole resistance in *Aspergillus fumigatus Antimicrob Agents Chemother,* 41**,** 1364-1368.

ESCRIBANO, P., PELÁEZ, T., MUÑOZ, P., BOUZA, E. & GUINEA, J. 2013. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? *Antimicrobial Agents and Chemotherapy,* 57**,** 2815-2820.

GONÇALVES, V. N., CANTRELL, C. L., WEDGE, D. E., FERREIRA, M. C., SOARES, M. A., JACOB, M. R., OLIVEIRA, F. S., GALANTE, D., RODRIGUES, F., ALVES, T. M., ZANI, C. L., JUNIOR, P. A., MURTA, S., ROMANHA, A. J., BARBOSA, E. C., KROON, E. G., OLIVEIRA, J. G., GOMEZ-SILVA, B., GALETOVIC, A., ROSA, C. A. & ROSA, L. H. 2016. Fungi associated with rocks of the Atacama Desert: taxonomy, distribution, diversity, ecology and bioprospection for bioactive compounds. *Environmental Microbiology,* 18**,** 232-245.

GYOTOKU, H., IZUMIKAWA, K., IKEDA, H., TAKAZONO, T., MORINAGA, Y., NAKAMURA, S., IMAMURA, Y., NISHINO, T., MIYAZAKI, T., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., YASUOKA, A., YAGUCHI, T., OHNO, H., MIYZAKI, Y., KAMEI, K., KANDA, T. & KOHNO, S. 2012. A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. *Med Mycol.,* 50**,** 631-636.

HONG, S. B., GO, S. J., SHIN, H. D., FRISVAD, J. C. & SAMSON, R. A. 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia,* 97**,** 1316-1329.

KIDD, S. E., GOEMAN, E., MEIS, J. F., SLAVIN, M. A. & VERWEIJ, P. E. 2015. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses,* 58**,** 350-355.

NOVÁKOVÁ, A., HUBKA, V., DUDOVÁ, Z., MATSUZAWA, T., KUBÁTOVÁ, A., YAGUCHI, T. & KOLAŘÍK, M. 2014. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. *Fungal Diversity.,* 64**,** 253-274.

PATTERSON, T. F., THOMPSON III, G. R., DENNING, D. W., FISHMAN, J. A., HADLEY, S., HERBRECHT, R., KONTOYIANNIS, D. P., MARR, K. A., MORRISON, V. A., HONG NGUYEN, M., SEGAL, B. H., STEINBACH, W. J., STEVENS, D. A., WALSH, T. J., WINGARD, J. R., YOUNG, J. A. H. & BENNETT, J. E. 2016. Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. *Clinical Infectious Diseases,* 63**,** e1-e60.

PELÁEZ, T., ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., VALERIO, M., BLANCO, J. L., GARCIA, M. E., MUÑOZ, P., CUENCA-ESTRELLA, M. & BOUZA, E. 2013. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. *Journal of Medical Microbiology,* 62**,** 474-478.

POSTERARO, B., MATTEI, R., TRIVELLA, F., MAFFEI, A., TORRE, A., DE CAROLIS, E., POSTERARO, P., FADDA, G. & SANGUINETTI, M. 2011. Uncommon Neosartorya udagawae fungus as a causative agent of severe corneal infection. *J Clin Microbiol.,* 49**,** 2357-2360.

SAMSON, R. A., HONG, S., PETERSON, S. W., FRISVAD, J. C. & VARGA, J. 2007. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Studies in Mycology,* 59**,** 147-203.

SAMSON, R. A., VISAGIE, C. M., HOUBRAKEN, J., HONG, S. B., HUBKA, V., KLAASSEN, C. H., PERRONE, G., SEIFERT, K. A., SUSCA, A., TANNEY, J. B., VARGA, J., KOCSUBÉ, S., SZIGETI, G., YAGUCHI, T. & FRISVAD, J. C. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Studies in Mycology,* 78**,** 141-173.

SUGUI, J. A., PETERSON, S. W., FIGAT, A., HANSEN, B., SAMSON, R. A., MELLADO, E., CUENCA-ESTRELLA, M. & KWON-CHUNG, K. J. 2014. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J Clin Microbiol.,* 52**,** 3707-3721.

SUGUI, J. A., VINH, D. C., NARDONE, G., SHEA, Y. R., CHANG, Y. C., ZELAZNY, A. M., MARR, K. A., HOLLAND, S. M. & KWON-CHUNG, K. J. 2010. *Neosartorya udagawae* (*Aspergillus udagawae*), an emerging agent of aspergillosis: how different is it from *Aspergillus fumigatus*? *Journal of Clinical Microbiology,* 48**,** 220-228.

TALBOT, J. J., JOHNSON, L. R., MARTIN, P., BEATTY, J. A., SUTTON, D. A., BILLEN, F., HALLIDAY, C. L., GIBSON, J. S., KIDD, S., STEINER, J. M., UJVARI, B. & BARRS, I. R. 2014. What causes canine sino-nasal aspergillosis? A molecular approach to species identification. *The Veterinary Journal,* 200**,** 17-21.

TAMIYA, H., OCHIAI, E., KIKUCHI, K., YAHIRO, M., TOYOTOME, T., WATANABE, A., YAGUCHI, T. & KAMEI, K. 2015. Secondary metabolite profiles and antifungal drug susceptibility of *Aspergillus fumigatus* and closely related species, *Aspergillus lentulus, Aspergillus udagawae,* and *Aspergillus viridinutans*. *Journal of Infection and Chemotherapy,* 21**,** 385-391.

VINH, D. C., SHEA, Y. R., JONES, P. A., FREEMAN, A. F., ZELAZNY, A. & HOLLAND, S. M. 2009a. Chronic invasive aspergillosis caused by *Aspergillus viridinutans*. *Emerg Infect Dis.,* 15**,** 1292-1294.

VINH, D. C., SHEA, Y. R., SUGUI, J. A., PARRILLA-CASTELLAR, E. R., FREEMAN, A. F., CAMPBELL, J. W., PITTALUGA, S., JONES, P. A., ZELAZNY, A., KLEINER, D., KWON-CHUNG, K. J. & HOLLAND, S. M. 2009b. Invasive aspergillosis due to *Neosartorya udagawae*. *Clinical Infectious Diseases,* 49**,** 102-111.

Chapter 2. Literature Review

2.1 Aspergillosis: a one health infectious disease

2.1.1 Aspergillosis and its host species

Aspergillosis is the infection of a host by an opportunistic fungal pathogen of the genus *Aspergillus*. It is a one health infectious disease, causing a range of conditions in a variety of animal species including humans, including acute and chronic, non-invasive and invasive forms. Infection is most commonly attributed to fungi in *Aspergillus* section *Fumigati*, and most frequently to the fungus *Aspergillus fumigatus* sensu stricto (Vanden Bossche et al., 1988, Bodey and Vartivarian, 1989, Kurup and Kumar, 1991, Dixon and Walsh, 1992, Latgé et al., 1997, Balajee et al., 2009b, Alastruey-Izquierdo et al., 2013, Negri et al., 2014), a ubiquitous saprophyte common in soil, compost and spread via air (Mullins et al., 1976, Mullins et al., 1984, Vanden Bossche et al., 1988, Pitt, 1994, Haines, 1995). Aspergillosis is primarily a disease of the respiratory tract, generally following inhalation of environmental spores (Latgé, 1999). Aspergillosis develops when the host's immune system cannot remove *Aspergillus* spores due to either an overwhelming fungal load and/ or immunosuppression. Aspergillosis causes significant morbidity amongst infected hosts, with a high mortality rate in invasive aspergillosis (IA), presenting diagnostic and treatment challenges to clinicians.

Aspergillosis has been most widely reported in humans, where invasive disease most commonly affects immunocompromised individuals. Immunocompromise can be secondary to other diseases (e.g. acquired immunodeficiency virus, leukaemia) or immunosuppressive therapies (e.g. corticosteroids, chemotherapy) (Nash et al., 1997, Denning, 1998, Meersseman et al., 2004, Shaukat et al., 2005, Chamilos et al., 2006, Stergiopoulou et al., 2007, Segal and Romani, 2009, Balajee et al., 2009b, Neofytos et al., 2009, Kontoyiannis et al., 2010, Pappas et al., 2010). Chronic pulmonary aspergillosis (CPA) is most commonly reported in patients with current or prior lung disease, including cystic fibrosis (CF), tuberculosis and asthma (Laufer et al., 1984, Greenberger and Patterson, 1988, Denning, 2009, Nam et al., 2010, Denning et al., 2011b, Denning et al., 2016). In humans, the disease has no identified predilection for age or gender.

Natural infection includes reports in land and marine mammals, birds and reptiles. Disease is most commonly reported in stressed or immunocompromised captive birds exposed to high environmental burdens of fungal spores, attributed to husbandry practices and facility location (Balseiro et al., 2005, Burco et al., 2012, Neumann, 2016). Various avian species have been reported to be affected, with no species predilection. This is similar to horses, where host immunosuppression predisposes to aspergillosis associated with different diseases including neoplasia, endocrinopathies and enteritis (Hattel et al., 1991, Carrasco et al., 1996). In contrast, breed predilections have been identified in dogs and cats indicating a genetic factor in disease susceptibility. In dogs, infection occurs most commonly in medium or long nosed breeds (mesaticephalic or dolicocephalic) (Peeters and Clercx, 2007), whereas in cats short-nosed breeds of oriental descent are over represented (Barrs et al., 2015). Additionally, aspergillosis often affects systemically immunocompetent, young to middle-aged canine and feline patients (Sharp et al., 1991, Barrs and Talbot, 2014). Reports of aspergillosis in ruminants are mostly associated with increased environmental burdens, particularly contaminated feed, with increased disease susceptibility in recently calved and early lactating cows (Puntenney et al., 2003). Naturally occurring aspergillosis is reported in many other species including rhinoceros, dolphins, whales, rabbits, turtles, tortoises, snakes, chuckwallas, crocodiles and non-human primates (Scott, 1930, Frye and Dutra, 1974, Tappe et al., 1984, Joseph et al., 1986, Domingo et al., 1992, Buenviaje et al., 1994, Reidarson et al., 1998, Woods et al., 1999, Miller et al., 2004, Myers et al., 2009, Prahl et al., 2011, Delaney et al., 2013, Bryant et al., 2016, Allizond et al., 2016, Cassle et al., 2016, Groch et al., 2018), mostly due to host immune-compromise and poor husbandry.

2.1.2 Forms and agents of disease

Invasive and non-invasive forms of aspergillosis occur (Table 1). Locally or systemically invasive infections have a high mortality rate as they often lead to dissemination of the fungus via the circulatory system. Localised disease is often chronic and usually confined to the upper and/or lower respiratory tract. Most of the wide-ranging types of *Aspergillus* diseases

in natural infections have also been reported in humans, including respiratory, otic, ocular, cutaneous and disseminated disease (Latgé, 1999, Parize et al., 2009, Chrdle et al., 2012, Denning et al., 2016, Patterson et al., 2016, Chowdhary et al., 2017).

In humans, aspergillosis can present as allergic, chronic or invasive disease mostly involving the respiratory system. Allergic *Aspergillus* conditions include severe asthma with fungal sensitisation and allergic bronchopulmonary aspergillosis (ABPA) (Chowdhary et al., 2017). CPA, mostly in immunocompetent patients, has been associated with ABPA, cystic fibrosis and other chronic granulomatous diseases such as tuberculosis (Laufer et al., 1984, Greenberger and Patterson, 1988, Denning, 2009, Nam et al., 2010, Denning et al., 2011b, Denning et al., 2016). IA, however, mostly affects severely immunocompromised, including transplant patients (solid organ and haematopoietic stem cell transplant patients) and patients on immunosuppressive therapies, such as corticosteroids and chemotherapy (Nash et al., 1997, Denning, 1998, Meersseman et al., 2004, Shaukat et al., 2005, Hope et al., 2005, Chamilos et al., 2006, Stergiopoulou et al., 2007, Segal and Romani, 2009, Balajee et al., 2009b, Neofytos et al., 2009, Kontoyiannis et al., 2010, Pappas et al., 2010). The recently described *Aspergillus* bronchitis occurs in patients with cystic fibrosis, bronchiectasis, lung transplant recipients and patients in intensive care units on mechanical ventilation (Chrdle et al., 2012).

Localised (invasive and non-invasive) and disseminated (invasive) infections have been reported in captive and companion animal species. The respiratory tract is mostly affected in dogs, cats, birds and horses, where bronchopulmonary, sino-nasal and sino-orbital infections are described, as well as air sacculitis in birds and guttural pouch infection in horses (Ainsworth and Rewell, 1949, Cook, 1968, Burr, 1981, Harvey, 1984, Dyar et al., 1984, Southard, 1987, Lane, 1989, Sharp et al., 1991, Tsai et al., 1992, Tasker et al., 1999, Cork et al., 1999, Willis et al., 1999, Hamilton et al., 2000, des Lions et al., 2000, Zonderland et al., 2002, Akan et al., 2002, Kim et al., 2003, Perez et al., 2003, Peeters et al., 2005, Whitney et al., 2005, Balseiro et al., 2005, Vestappen and Dorrestein, 2005, Tell, 2005, Mathews and Sharp, 2006, Johnson et al., 2006, Nardoni et al., 2006, Peeters and Clercx, 2007, Barrs et al., 2007, Xavier et al., 2007, Meler et al., 2008, Kano et al., 2008, Zafra et al., 2008, Kendall et al., 2008, Billen et al., 2009a, Day, 2009, Cacciuttolo et al., 2009, Barachetti et al., 2009, Sharman et al., 2010, Smith and Hoffman, 2010, Sato and Itagaki, 2010, Adamama- Moraitou et al., 2011, Ito et al., 2011, Hazell et al., 2011, Arne et al., 2011, Barrs et al., 2012, Dobesova et al., 2012, Kano et al., 2013, Barrs et al., 2013, Barrs et al., 2014, Barrs et al., 2015,

Tamborini et al., 2016, de Souto et al., 2016, Neumann, 2016). Infection of the reproductive system is common in cows, including mastitis and placentitis, a cause of mycotic abortion (Knudtson and Kirkbride, 1992, Pérez et al., 1998, Las Heras et al., 2000, Puntenney et al., 2003, Garcia et al., 2004). The most frequently reported infections in reptiles are cutaneous (Frye and Dutra, 1974, Jacobson et al., 2000, Miller et al., 2004, Girling and Fraser, 2009), and in marine mammals, encephalitis is described (Domingo et al., 1992, Dagleish et al., 2006, Barley et al., 2007, Dagleish et al., 2008). Other forms of disease are reported in many species including otologic (Coyner, 2010, Prahl et al., 2011), dermatologic (Chute et al., 1955, Mohammed et al., 1980, Tappe et al., 1984, Abrams et al., 2001, Suedmeyer et al., 2002), ocular (Pickett et al., 1985, Beckman et al., 1994, Hoppes et al., 2000, Abrams et al., 2001, Sansom et al., 2005, Labelle et al., 2009, Dalton and Ainsworth, 2011, Wada et al., 2013), gastrointestinal (Jensen et al., 1994, Puntenney et al., 2003) and musculoskeletal systems (van Veen et al., 1999, Cortes et al., 2005, Olias et al., 2010, Simpson, 2011, Barnett et al., 2011), and disseminated infection (Scott, 1930, Sautter et al., 1955, Schiefer, 1965, Pakes et al., 1967, Weiland, 1970, McCausland,
1972, Bolton and Brown, 1972, Stokes, 1973, Vogler and Wagner, 1975, Fox et al., 1978, Köhler et al., 1978, Kirkpatrick, 1982, Migaki, 1983, Pickett et al., 1985, Ossent, 1987, Severo et al., 1989, Jensen et al., 1990, Burk et al., 1990, Reidarson et al., 1999, Pérez et al., 1999, Adamama-Moraitou et al., 2001, Throne Steinlage et al., 2003, Hope et al., 2005, Martin et al., 2007, Schultz et al., 2008, Hunter and Nation, 2011, Hazell et al., 2011, Dégi et al., 2011, Jurczynski et al., 2012, Zhang et al., 2012, Abdo et al., 2012, Groch et al., 2018).

Historically *A. fumigatus* has been the most frequently identified cause of aspergillosis in humans. Traditionally, fungal cultures were identified in the clinical setting based on morphology following mycelial and/ or conidial growth on agar. Other methods included identifying fungal elements such as conidia on cytological examination of bodily fluids and aspirates or identifying fungal spores and inflammatory cells on histopathology (McClenny, 2005).

The introduction of molecular identification techniques in the past 20 years has demonstrated that morphological identification alone often cannot distinguish between similar species of *Aspergillus* (Hong et al., 2005, Samson et al., 2007, Balajee et al., 2007, Balajee et al., 2009a, Lamoth, 2016). These are the so-called "cryptic" *Aspergillus* species. Retrospective and prospective epidemiological studies employing molecular

techniques for clinically relevant *Aspergillus* isolates identified new and existing *Aspergillus* fungal species as opportunistic pathogens. While *A. fumigatus* is still the most common cause of aspergillosis in humans, 18 other *A.* sect. *Fumigati* species are now reported as pathogens (Guarro et al., 2002, Sutton et al., 2002, Balajee et al., 2005a, Balajee et al., 2005b, Balajee et al., 2006, Alhambra et al., 2008, Balajee et al., 2009b, Vinh et al., 2009a, Vinh et al., 2009b, Sugui et al., 2010, Posteraro et al., 2011, Coelho et al., 2011, Gyotoku et al., 2012, Alastruey-Izquierdo et al., 2013, Barrs et al., 2013, Peláez et al., 2013, Toskova et al., 2013, Malejczyk et al., 2013, Sugui et al., 2014, Negri et al., 2014, Alvarez-Pérez et al., 2014, Khare et al., 2014, Frisvad and Larsen, 2016). Molecular identification also identified *A. fumigatus* as the most common cause of sino-nasal infection in cats and dogs; however sino-orbital infection in cats is mostly due to members of the *Aspergillus viridinutans* species complex (AVSC) (Kano et al., 2008, Barrs et al., 2012, Barrs et al., 2013, Kano et al., 2013, Barrs et al., 2014). Traditional morphological identification is reported for most other animal- associated *Aspergillus* species, with molecular identification studies lacking for many.

Table 1. Types of aspergillosis reported in natural infections

Host	**Invasive disease**	**Non-invasive disease**
Human	Invasive pulmonary aspergillosis, disseminated, invasive bronchial aspergillosis, chronic invasive pulmonary aspergillosis (necrotising, cavitary, fibrosing), acute/ chronic invasive sinusitis, sino-orbital aspergillosis, cerebral aspergillosis, CNS aspergillosis, chronic granulomatous sinusitis, cardiovascular aspergillosis, osteomyelitis, septic arthritis, endopthalmitis, renal aspergillosis, orchitis, prostatic aspergillosis, pelvic aspergillosis, gastrointestinal aspergillosis	Aspergilloma ("fungus ball") of the sinus, lung or kidney, allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis, keratitis, scleritis, cutaneous aspergillosis
Canine	Disseminated, bronchopulmonary, sino-orbital aspergillosis	Sino-nasal aspergillosis, otomycosis
Feline	Sino-orbital aspergillosis, bronchopulmonary aspergillosis, disseminated	Sino-nasal aspergillosis, ulcerative keratomycosis
Avian	Invasive pulmonary aspergillosis, disseminated, spondylitis, articular aspergillosis (hip joint), pulmonary aspergilloma	Air-sacculitis, blepharitis, keratitis, keratoconjunctivitis, dermatitis, omphalitis, nasal aspergillosis
Equine	Invasive pulmonary aspergillosis, encephalitis	Guttural pouch mycosis, sino-nasal aspergillosis, keratitis
Bovine	Mycotic placentitis, bronchopulmonary, gastroenteritis, mastitis, lymphadenitis	
Non-human primate	Disseminated	
Reptile	Disseminated, bronchopulmonary	dermatitis, keratitis
Marine mammal	Disseminated, encephalitis, otitis media/ otits interna, invasive pulmonary aspergillosis	

2.1.3 Diagnosis

Ante-mortem diagnosis of aspergillosis can be challenging for clinicians with patients often presenting with non-specific clinical signs. Following a history and presentation that alerts the clinician to the possibility of fungal infection, several tests can confirm or support a diagnosis of aspergillosis. Sometimes however, diagnosis is only achieved post-mortem.

In human patients, diagnosis is based on imaging (radiographs, computed tomography (CT), magnetic resonance imaging (MRI), bronchoscopy), observation of fungal hyphae in tissue biopsy or fluid sampling and analysis (sputum, bronchoalveolar lavage (BAL) fluid, cerebrospinal fluid, lesion aspirates), with or without positive fungal culture, serology (galactomannan and β-D-glucan assay) and histopathology (Patterson et al., 2016). More recently, molecular detection of *Aspergillus* fungi in sputum has proven more sensitive than sputum culture to diagnose aspergillosis in cystic fibrosis patients (Guegan et al., 2018). The sensitivity and specificity of serological tests for aspergillosis varies within and between patient populations and for antigen versus antibody tests. Results from the β-D-glucan antigen test can support a positive culture; however sensitivity and specificity varies greatly amongst IA patients with haematologic malignancies (Odabasi et al., 2004, Kawazu et al., 2004, Ostrosky-Zeichner et al., 2005, Pickering et al., 2005, Obayashi et al., 2008, Koo et al., 2009). Another antigen test, galactomannan, is most useful to diagnose IA in patients with haematopoietic cell transplants or haematologic malignancies (Mennink-Kersten et al., 2004, Marr et al., 2005). *Aspergillus* antibody detection is useful in immunocompetent patients with aspergilloma, ABPA or CPA, due to the significant humoral response to *Aspergillus* antigen (Latgé, 1999, Barton et al., 2008, Guitard et al., 2012, Ohba et al., 2012). Antibodies are usually detected via gel antibody-antigen complexes with the predominant precipitation of immunoglobulin G (IgG), achieved by immunodiffusion or counter-immunoelectrophoresis or via enzyme linked immunosorbent assay (ELISA) to detect IgG (Page et al., 2015).

Non-specific clinical signs can make ante-mortem veterinary diagnosis difficult and diagnosis is often post-mortem via observing fungal plaques or granulomas in tissues, confirmed by histopathology and positive fungal culture.

Definitive ante-mortem diagnostic tests depend on host species but include imaging (radiographs, CT scans, endoscopy), positive culture from biopsy, cytology and histopathology (Jenkins, 1991, Redig, 1994, Phalen, 2000, Jones and Orosz, 2000, Saunders and van Bree, 2003, Saunders et al., 2004, Schultz et al., 2008, Barrs et al., 2012, Barrs et al., 2014). Serology also has limited diagnostic success in animals, dependent on the immunocompetence of the host, but is useful in some species, including antigen and antibody testing in some avian species with respiratory or disseminated aspergillosis, and antigen testing in dogs with disseminated IA (Jones and Orosz, 2000, Deem, 2003, Billen et al., 2009b, Cray et al., 2009a, Cray et al., 2009b, Franca et al., 2012, Garcia et al., 2012). In cats and dogs with SNA or SOA, antibody detection can be useful with a demonstrated sensitivity and specificity >90% in cats, and specificity >90% and sensitivity >76% in dogs (Barrs et al., 2015, Taylor et al., 2016, Billen et al., 2009b). Positive culture of *Aspergillus* from non-sterile sites must be supported by additional diagnostic tests.

Mycology researchers use a polyphasic taxonomic approach to analyse species' relatedness and describe new species. This includes morphological analysis (macro and micro morphological features), growth conditions / characteristics (agar types, temperatures of growth, degree of sporulation), phylogenetic analysis of multiple genes (multi-locus sequencing) and secondary metabolite profiling of fungal specimens (Hong et al., 2005, Samson et al., 2007). The most commonly targeted genes for phylogeny are the ITS, BenA and calmodulin genes, with RPB2, actin, TSR1, elongation factor and MCM7 also used (White et al., 1990, Glass and Donaldson, 1995, Hong et al., 2005, Sugui et al., 2014). This full array of laboratory techniques is rarely available to identify species in a fungal culture in the clinical setting. Traditional morphological methods are still the main identification method in reference laboratories; however adding at least molecular identification of fungal isolates when a cryptic species is suspected or when antifungal susceptibility profiling returns high or resistant results is becoming more common. Amplification of the ITS region for identification to species complex level and either the calmodulin or partial beta- tubulin genes for identification to species level are the most commonly targeted genes and are recommended by the International Society for Human and Animal Mycology (ISHAM) (Powers-Fletcher and Hanson, 2016). Some centres also use

matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) for species level identification (Lestrade et al., 2016).

2.1.4 Treatment and prognosis

Most *A.* sect. *Fumigati* species are resistant to certain azole drugs (fluconazole, ketoconazole) and 5-flucytosine C. They are, however, generally susceptible to the newer classes of triazole drugs (posaconazole, voriconazole), echinocandins (caspofungin, anidulafungin and micafungin) and amphotericin-B. Some cryptic species are less susceptible to triazoles (Alcazar-Fuoli et al., 2008, Escribano et al., 2013, Alastruey-Izquierdo et al., 2014).

Azole antifungal therapeutics are the first line treatment of aspergillosis. Azoles target the production of sterol synthesis, an important component of the fungal cell membrane and fungal cell growth (Hargrove et al., 2015). Azole drug selection depends on the form of disease (i.e. invasive vs localized, non-invasive), when performed (e.g. in patients unresponsive to therapy), and *in vitro* antifungal susceptibility results (Patterson et al., 2016). Additionally, drug withholding periods for production animals, cost and availability affect treatment choice, and with advanced disease euthanasia is often inevitable. Prognostic factors include the fungal species causing infection, its antifungal susceptibilities, disease form and location, and the host's immune response (Verweij et al., 2015, Patterson et al., 2016).

Removing fungal balls from humans with chronic fungal rhinosinusitis via endoscopic surgery generally resolves infection (Uri et al., 2003). The triazole drugs itraconazole, voriconazole, posaconazole and isavuconazole are the oral drugs of choice for treating invasive and chronic forms of aspergillosis in humans, and posaconazole, voriconazole and isavuconazole are also available as intravenous preparations (Patterson et al., 2016, Ullmann et al., 2018). Voriconazole and isavuconazole are the first-line treatments of choice for IA (Walsh et al., 2008, Patterson et al., 2016, Ullmann et al., 2018). Other non-triazole intravenous drugs (liposomal amphotericin B and echinocandins) are usually only suitable for IA cases that do not respond to azole therapy (i.e. salvage therapy), where azoles cannot be administered, or, as for liposomal amphotericin B, as the first-line treatment of IA in neonates and where a high prevalence of

ARAf is known for an area or institution (Lestrade et al., 2016, Patterson et al., 2016, Ullmann et al., 2018).

IA, however, carries a high mortality rate of 28.5% to 50% (Bitar et al., 2014, Verweij et al., 2015). Many factors contributed to this mortality rate, including delays in diagnosis due to a lack of rapid and reliable diagnostic tests, and co-morbidities. Triazole treatment has reduced the mortality rate for IA to 19-20% (Neofytos et al., 2009, Verweij et al., 2015, Maertens et al., 2016). However, mortality rates of up to 88% are reported due to the emergence of azole-resistant *A. fumigatus* (ARAf) strains (van der Linden et al., 2011, Steinmann et al., 2015). One Dutch study reported a high rate of ARAf in ICU patients with IA, with a 100% mortality rate (Russcher et al., 2014). Consequently, in areas (including regions and institutions / departments) where the prevalence of azole resistant strains of *A. fumigatus* is considered high (>10%), recommended alternate treatments include voriconazole and echinocandin combination therapy or liposomal amphotericin B monotherapy (Verweij et al., 2015).

Azole treatment for aspergillosis can be topical (imidazole class drugs enilconazole, clotrimazole, miconazole), systemic (triazole class drugs itraconazole, voriconazole, posaconazole), or both and used as monotherapy or combined with other antifungal drugs, including amphotericin B, terbinafine and echinocandins. Additionally, these drugs can be used without azoles as monotherapy or in combination (Seyedmousavi et al., 2015). Long term azole therapy is used in patients with chronic infections. Surgical debridement of fungal plaques and granulomas is also performed in cases of avian, canine and feline infections, and, in combination with topical azole application, is the current recommended treatment for dogs and cats with non-invasive sino-nasal aspergillosis (SNA), with systemic therapy only recommended when topical azoles cannot be administered (e.g. severe lysis of the cribriform plate) (Goodall et al., 1984, Zonderland et al., 2002, Tomsa et al., 2003, Claeys et al., 2006, Xavier et al., 2007, Furrow and Groman, 2009, Billen et al., 2010, Barrs et al., 2012, Tamborini et al., 2016, Stengel, 2017, Vedrine and Fribourg-Blanc, 2018). Medical and surgical therapies are required in horses with guttural pouch aspergillosis to prevent arterial haemorrhage (Church et al., 1986, Léveillé et al., 2000, Lepage and Piccot-Crezollet, 2005, Delfs et al., 2009). Generally, for all animal species, disseminated invasive aspergillosis carries a poor to grave prognosis; however, a high rate of treatment failure is also seen in localised, even non-invasive conditions, such as canine SNA where a mortality rate of 13% has

been reported and up to 70% of dogs require multiple treatments to achieve therapeutic success (Sharman et al., 2010). The pharmacokinetics of antifungal drugs have not been established for many animal species, and recommended doses have often come from few case reports.

2.2 *Aspergillus viridinutans* complex species

2.2.1 Background

Cryptic species are difficult to distinguish from more commonly identified fungi based on morphological analysis alone. Within *A.* sect. *Fumigati* this term refers to isolates morphologically similar to *A. fumigatus.* They are of medical importance and have been identified in prevalence studies as agents causing disease in human and veterinary patients. Many cryptic species infections are associated with high antifungal susceptibility profiles and less favourable prognoses. Thus, accurately identifying cryptic *Aspergillus* species is important for clinical treatment and determining prognosis.

The following paper is a literature review of the AVSC, a group of cryptic fungi in *A.* sect. *Fumigati*. It investigates this group's association with human and animal disease, environmental niches, and virulence traits (physiology, secondary metabolite production and antifungal susceptibility profiles) that can contribute to pathogenesis. This fungal group is important medically as most of the AVSC species have been associated with invasive disease in humans and animals that is refractory to treatment and is mostly fatal. This article reviews the current literature on members of the AVSC.

2.2.2 Publication

The following publication has been re-formatted here to suit the style of this thesis. The publication's in-text references remain in the style required by the journal (superscript number form) and the publication associated tables, figures, references and supplementary data follow the main text. This research is presented in its original publication form in **Appendix 1**. A list of all other references referred to in this chapter outside of section **2.2.2 Publication** can be found at the chapter's end (see **2.4 References**).

Talbot JJ, Barrs VR. One-health pathogens in the *Aspergillus viridinutans* complex. *Medical Mycology*. Volume 56, Issue 1, 1 January 2018, Pages 1–12, https://doi.org/10.1093/mmy/myx016.

Abstract

Cryptic species in *Aspergillus* section *Fumigati* are increasingly recognised as pathogens in humans and animals. The *A. viridinutans* complex (AVC) has recently expanded to comprise 10 species, of which six are known to be pathogenic, including *A. udagawae, A. felis, A. pseudofelis, A. parafelis, A. pseudoviridinutans,* and *A. wyomingensis.* They cause locally invasive and disseminated invasive disease syndromes, including chronic pulmonary aspergillosis and invasive aspergillosis in humans, invasive fungal rhinosinusitis in cats, and disseminated invasive aspergillosis in dogs. In contrast to *A. fumigatus*, AVC species are characterized by higher minimum inhibitory concentrations (MICs) of antifungal drugs and the infections they cause are typically more chronic and more refractory to therapy. This review, of relevance for one-health practitioners, explores the history of the AVC as well as current phylogenetic relationships, secondary metabolite production, environmental distribution, clinical syndromes, and antifungal susceptibility patterns.

Introduction

Aspergillus section *Fumigati* contains 63 species, 19 of which are known human and veterinary pathogens (Fig. 1).[1,2] Since the implementation of polyphasic taxonomical methods for fungal identification, there has been a striking increase in the number of cryptic and newly discovered species of medical importance within the section. Cryptic species can be defined as isolates of closely related fungal species that are indistinguishable on classical morphological features but can be identified to species level using molecular techniques.[3] The most common cryptic species in section *Fumigati* associated with clinical disease in humans are *A. lentulus*,[4–18] *A. thermomutatus*,[11,19,20] and members of the *A. viridinutans* complex (AVC).[6,21–26] Of the 10 species within this complex, six have been identified by multi-locus sequencing as pathogens in humans and animals including *A. udagawae, A. felis, A. pseudofelis, A. parafelis, A. pseudoviridinutans*, and *A. wyomingensis*. They cause locally invasive and disseminated invasive disease syndromes, have increased resistance to commonly used antifungal therapies, and are associated with high mortality rates.[4,21–27] Although *A. viridinutans sensu stricto* has

been reported as a pathogen, this is questionable as isolates in these reports were identified using morphological methods or only one gene was sequenced for phylogeny.[25–30] Some of these isolates have since been definitively identified and confirmed to be other species within the complex.[4,24,25,31] Three other species within the AVC, *A. aureoles, A. arcoverdensis*, and *A. siamensis* have been isolated from environmental samples only and their pathogenic potential is not known.[31–36] This review explores the history of the AVC and highlights the need for medical and veterinary practitioners to be aware of this important group of closely related fungi. Morphological features, secondary metabolite production, current phylogenetic relationships, clinical syndromes, and antifungal susceptibility patterns of the ten currently accepted members are discussed.

Species in the A. viridinutans complex and their characteristics

There are currently ten accepted species within the AVC: *A. viridinutans sensu stricto, A. udagawae, A. felis, A. wyomingensis, A. aureolus, A. parafelis, A. pseudofelis, A. pseudoviridinutans, A. siamensis*, and *A. arcoverdensis* (Fig. 1).[4,24,31,32,34–39]*Aspergillus viridinutans sensu stricto* was discovered in 1954 in the Australian environment.[39] This was shortly followed by the discovery of *Aspergillus aureolus*,[32] previously known as *Sartorya aureola*,[40]*Neosartorya aureola*[41] and *A. aureoluteus.*[42] It was not until the mid-1990s that another species, *Neosartorya udagawae* (now *A. udagawae*) was described and added to the complex.[34] In recent years the complex has further expanded. Since 2013 seven more species have been described, largely attributed to advances in polyphasic taxonomy, which utilizes morphologic, physiologic, and molecular characteristics to determine isolate speciation and phylogeny.[37,43]

Nomenclature changes

In 2011 the International Association for Plant Taxonomy (IAPT) adopted changes to the rules for naming fungi. A one fungus–one name approach was adopted, enforcing the adoption of the same genus name for teleomorph (sexual) and anamorph (asexual) types of a species.[44] This meant the sexual state of fungi previously known as *Neosartorya* was to be replaced with *Aspergillus*. Changes were also made around the Latin designation of a species epithet. The termination of a name is dependent on whether the epithet is based on gender, geography, if there is an existing Latin name and species basionyms, as per Chapter III, Section 4 to Chapter

IX, Section 2 of the International Code of Nomenclature for algae, fungi, and plants.[44] In cases Where the names of species had been changed from their original name over time, names reverted to the original name. Thus, *Neosartorya udagawae* became *Aspergillus udagawae*, while *Aspergillus aureolus* maintained its basionym with no other variations.

Species in the AVC share some common morphological features. They are typically slow growing, slow to sporulate and thermophilic with optimal growth between 35° and 42°C.[4,24,31,32,34–39]*Aspergillus aureolus* and *A. siamensis* are the only homothallic species in the complex.[31,36]

Mating genotype and phenotype has been determined for *A. felis, A. wyomingensis, A. udagawae,* and *A. parafelis*.[4,24,31] Mating genotype, but not phenotype, has been confirmed in *A. viridinutans sensu stricto, A. pseudofelis, A. pseudoviridinutans,* and *A. arcoverdensis*.[4,31,35] Fertile interspecies matings have been reported between *A. parafelis* with *A. fumigatus sensu stricto, A. viridinutans sensu stricto, A. felis, A. pseudoviridinutans* and *A. wyomingensis,* and also between *A. pseudofelis* with *A. felis.*[4]

It is difficult to distinguish AVC species from other species in section *Fumigati* based on morphological features alone. Although members possess characteristic "nodding heads" on cytological examination (Fig. 2), this feature occurs in some other section *Fumigati* species.[31] The inability to grow at 50°C can be exploited as a practical method in clinical laboratories to distinguish cryptic species in section *Fumigati* from *A. fumigatus sensu stricto*.[4,24,31] Distinguishing between species within the AVC is also difficult based on anamorph and teleomorph morphological features. The gold standard for identification is through the addition of molecular identification to morphological techniques.

Molecular identification

Phylogenetic relationships within the AVC have been determined by comparative sequence analysis of multiple loci including the internal transcribed spacer region (*ITS*), beta-tubulin (*benA*), calmodulin (*caM*), rodlet A (*rodA*), minichromosome maintenance factor (*Mcm7*), a pre-RNA processing protein (*Tsr1*), and the second largest unit of RNA polymerase II (*RPB2*).[4,24,31,42,43,45–49] Comparative multilocus sequence analysis is the gold standard for molecular identification although no one gene is currently accepted as a stand-alone method

for identification. [33,38] The most commonly used genes that have been used for species descriptions are *benA* and *caM*. [4,24,31,35,36]

Secondary metabolites

Determination of secondary metabolites may be useful to predict pathogenicity where only environmental isolates exist for a species.[1] Viriditoxin is a secondary metabolite produced by all species in the AVC.[1,50] The role of viriditoxin in disease pathogenesis is unknown, since it is produced by both known pathogens and species of unknown pathogenicity. However, one study demonstrated lethality in mice following intraperitoneal administration (LD_{50} of 2.8 mg), as well as the ability to inhibit growth of several bacterial species (*Corynebacterium, Bacillus,* and *Streptococcus*).[50,51] Viriditoxin is also produced by one other species in section *Fumigati, Aspergillus denticulate*.[52]*Aspergillus brevipes* was also reported to produce viriditoxin; however, this was since confirmed to be present in a mixed culture with *A. viridinutans sensu stricto*.[37,53,54]

A number of novel bioactive compounds have been discovered through secondary metabolite extraction of AVC species. Some extrolites have demonstrated anti-cancer and antibacterial properties. [50,55–58] Recent investigations into secondary metabolite extracts from environmental *A. felis* isolates demonstrated *in vitro* activity against *Paracoccidiodes brasiliensis PB*18.[58] Extracts from a range of fungal genera were tested, with isolates of *A. felis* and *A. udagawae* demonstrating the greatest activity against *P. brasiliensis PB*18. Using reversed-phase HPLC, *A. felis* extracts were identified as pyripyropene A, aspochalasin E, and cytochalasins (rosellichalasin, cytochalasin Kasp, and cytochalasin E) as well as a number of unknown compounds. Rosellichalasin and cytochalasin E have proven cytotoxic activity against human tumour cells lines,[59] while aspochalasin E is active against the cancer cells murine melanoma B16-F10 and human colon carcinoma HCT-116 cells.[60] Secondary metabolites produced from clinical and environmental isolates of both *A. udagawae* and *A. viridinutans sensu stricto* include fumigaclavine C, helvolic acid, methyl-sulochrin, pyripyropene A and E and trypacidin. *Aspergillus udagawae* also produces fumigaclavine A and fumiquinazoline F/G, while *A. viridinutans sensu stricto* also produces fumagillin, fumitremorgins A and C, and pyripyropenes O and S.[61] However, in the study that characterized these secondary metabolites, the clinical isolates listed with *BenA* and *CaM* sequences available on GenBank[62] (*A. udagawae* IFM 5058, IFM 51744, IFM 53867, IFM 53868, IFM 54303; *A. viridinutans*

IFM 54303), only had identity similarities with *BenA* and *CaM* markers for *A. udagawae* (AF132226, AB748566) and *A. viridinutans sensu stricto* (AF134779, DQ534162)[38] type strains of 97–99%.[61] Therefore, additional comparative gene sequence analyses may be necessary for definitive species identification of these isolates.

Recently MALDI-ToF mass spectrometry has been utilized for rapid identification of *Aspergillus* sp., including differentiation of *A. fumigatus sensu stricto* from the cryptic species *A. lentulus*.[63] More work is required for accurate identification of species within the AVC. However, this is a promising technique that is already used in clinical microbiology laboratories worldwide.

Identification of isolates in the clinical laboratory

Given the clinical management implications, clinical microbiologists can utilize specific laboratory techniques to increase recognition of cryptic species in section *Fumigati*. Knowledge of the correct fungal species is clinically relevant due to *in vitro* and *in vivo* antifungal resistance to commonly used therapeutics associated with some cryptic species infections.[7,8,19,64,65] Accurate identification is also essential for epidemiological studies to ascertain the prevalence of cryptic species infections in hospital populations.[19,64,65] While polyphasic taxonomy is the gold standard for species delimitation, this is not practical in the time crucial setting of clinical disease. Accurate identification of clinical isolates causing aspergillosis can be achieved using comparative sequence analysis of the partial *BenA* and *CaM* genes in laboratories where molecular techniques are available.[4,24,31,35,36] Initial isolation on malt extract agar at 37°C is suggested as these species will generally sporulate within 7 days on this medium for microscopic analysis and DNA extraction.[24] The presence of a `nodding head' (Fig. 2) should alert clinical mycologists to the possibility of an AVC species; however, other section *Fumigati* isolates have also been reported to share this feature.[31] The incubation of an isolate at 50°C is usually sufficient to distinguish most isolates of *A. fumigatus*, which is thermoresistant, from AVC species, which generally do not grow at temperatures higher than 42–45°C.[3,24,31,32,34–39] Slow sporulation of an isolate and high MIC values of antifungals should alert clinicians and laboratory mycologists to the possibility of cryptic species infection, and trigger molecular testing for definitive species identification. In addition to comparative sequence analysis, multiplex polymerase chain reaction (PCR) and restriction fragment length

polymorphism (RFLP) are other molecular techniques with potential application for identification of clinical isolates.[6,66,67]

Environmental prevalence

Aspergillus viridinutans complex species have been isolated from a range of habitats, and most appear to have worldwide distribution, including North and South America, Europe, Africa, Northeast Asia, and Asia-Pacific (Table 1). Four species in the AVC (*A. arcoverdensis, A. aureolus, A. siamensis* and *A. viridinutans sensu stricto*) have only been found in the environment and are of unknown pathogenic status.[32,34–36,39,68] The other six species havebeen isolated from both environmental and clinical samples. Environmental isolates have been sourced predominantly from soil, as well as food, air, animal faeces, and desert rocks. *Aspergillus felis* has been isolated from a variety of substrates, reflecting the ability of this organism to survive in a range of environments, and it appears to be globally distributed.[24,31,55] To date Only small numbers of *A. parafelis, A. pseudofelis*, and *A. pseudoviridinutans* have only been reported, and their environmental distribution is unknown (Table 1). *Aspergillus arcoverdensis* has been isolated from arid, semi-desert soil, and forest soil, demonstrating widespread distribution and an ability to survive in diverse environments.[35] The diverse range of environments inhabited by members of this complex, coupled with their potential for virulence, highlights the importance for medical and veterinary practitioners to be aware of these species.

Clinical disease and antifungal resistance

Cases of aspergillosis due to AVC species are being increasingly reported amongst human and veterinary patients, reflecting the importance of a One-Health approach to these mycoses (Table 2). Six species are recognized as pathogens: *A. udagawae, A. felis, A. wyomingensis, A. pseudofelis, A. parafelis*, and *A. pseudoviridinutans*.[2,4,21,24] Previous reports included *A. viridinutans sensu stricto* as a cause of disease in people; however, multilocus molecular analysis of these strains revealed they were *A. felis, A. parafelis, A. pseudofelis,* or *A. pseudoviridinutans.*[4,24–27]

These infections fall within the scope of disease due to `cryptic' *Aspergillus* species in section *Fumigati*. In recent studies where multilocus comparative sequence analysis was used for

identification, the prevalence of cryptic *Aspergillus sp.* infections in section *Fumigati* among human patients was 3–5%.[8,19,64] One European study examined 162 *Aspergillus* section *Fumigati* strains from hospitals in Spain and identified six cryptic section *Fumigati* species causing aspergillosis (3.7%), including one AVC species.[64] The Transplant-Associated Infection Surveillance Network and the American Centers for Disease Control and Prevention, Atlanta investigated cryptic species prevalence among transplant recipients with invasive aspergillosis (*n* = 216) and showed that 68% of isolates were from section *Fumigati*, amongst which there was a cryptic species prevalence of 5%. *Aspergillus udagawae* caused 2% of section *Fumigati* infections overall, and 37.5% of cryptic *A.* section *Fumigati* sp. infections.[8] Another study of 133 *Aspergillus sp.* isolates from human patients in Brazil showed 19% were cryptic species in section *Fumigati* but none belonged to the AVC.[19]

In section *Fumigati*, cryptic species infections in humans are most commonly caused by *A. lentulus*,[4–18] *A. udagawae*,[6,21–23] *A. thermomutatus*,[11,19,20] and *A. felis*.[24,25] Two other species *A. fumigatiaffini*[11] and *A. novofumigatus*[14,26] have been isolated from clinical specimens, however further evidence to support their role as pathogens is required. Further prevalence data are also needed in the veterinary field, since there are few molecular based studies. One study found no cryptic species in section *Fumigati* in dogs with non-invasive fungal rhinosinusitis, which is the most common anatomic form of aspergillosis in this host.[69] This is in contrast to the high prevalence of cryptic section *Fumigati* species isolated from cases of fungal rhinosinusitis in cats (72%, *n* = 33/46), of which the majority (81%, *n* = 29/36) were AVC species.[2,24,70–74] clinical disease in dogs and cats occurs most commonly in systemically immunocompetent animals. Information gleaned from naturally infected animal hosts can be used to further knowledge across human and veterinary medicine, including agents of disease, their virulence, antifungal susceptibilities, disease progression, and management.

Human infections

Human infections caused by the AVC have been reported in Spain, Portugal, the Czech Republic, Italy, Norway, the United States, and Japan. The most common presentation is chronic invasive pulmonary aspergillosis in immunosuppressed patients, which can become disseminated.[4,21,24,26,27] *Aspergillus felis*, *A. parafelis*, and *A. udagawae* have been isolated from such cases.[4,21,24] One patient with leukaemia and *A. felis* infection was coinfected with *A. novofumigatus*.[26] Another case, caused by *A. parafelis*, was characterized by chronic spread

of infection across anatomical planes, including cervical and thoracic lymph nodes, lungs, and pleura.[4,27] In addition, invasive pulmonary aspergillosis due to *A. udagawae* has a more chronic disease course than that caused by *A. fumigatus sensu stricto*.[21]*Aspergillus udagawae* has also been reported to cause localised bronchial infection in an elderly immunosuppressed patient,[22] acute respiratory distress syndrome (ARDS) in a middle-aged immunocompetent patient,[75] and a severe corneal infection.[23] In a study investigating the prevalence of cryptic species among US and European reference laboratory collections of isolates morphologically identified as *A. fumigatus*, RFLP analysis, and multilocus sequence typing identified *A. udagawae* from brain, sputum, BAL, and lung samples.[11]

Other clinical isolates obtained from patients include *A. pseudofelis* from sputum and a nail, *A. parafelis* from oropharyngeal exudates, *A. pseudoviridinutans* from a mediastinal lymph node,[4] and *A. udagawae* from a nail.[31] The identity of some clinical isolates, reported to be *A. udagawae* or *A. viridinutans sensu stricto*, is likely to be inaccurate for at least some isolates, since some were based on nucleotide homology of <99% with available, most closely related type strain sequences, or only one gene region was sequenced or the sequence was not submitted to GenBank.[62] Such isolates require further identification, especially in light of the number of recently discovered new species in the *A. viridinutans* complex.[6,14,21,29,61,64,76–77]

Animal infections

The most commonly described syndrome caused by the AVC is sino-orbital aspergillosis (SOA) in cats, a form of chronic invasive fungal rhinosinusitis. Disease has been reported in three geographical regions: Europe, Asia, and Australia.[24,71–73] Infection is most commonly caused by *A. felis*, but *A. udagawae* and *A. wyomingensis* have also been isolated from affected cats.[2,24,72,73] While *A. viridinutans sensu stricto* was reported as a cause of feline infection, this is doubtful as its identity was based on sequencing of the partial beta-tubulin gene only and the closest match using the GenBank BLAST tool[62] was 99%.[73] Previous isolates showing a similar percentage homology to other members of this complex have been demonstrated to be different species.[4,24,31,71]

Feline SOA carries a poor prognosis and is often fatal.[24,70,71] Cats typically present with signs of an orbital mass (exophthalmos, prolapse of the nictitating membrane (third eyelid), exposure keratitis and conjunctival hyperaemia), and signs or a recent history of nasal disease (sneezing,

nasal discharge, epistaxis). Extension of disease beyond the orbit often results in an oral mass or ulcer in the pterygopalatine fossa and paranasal soft tissue swelling. Mild mandibular lymphadenopathy is also common. In advanced disease, neurological signs including hyperaesthesia and seizures may be present.[24,70,71] Infection due to A. *felis* has also been reported in a dog with disseminated invasive aspergillosis.[24]

It is currently unclear why *A. felis* is a major fungal pathogen of cats but is rarely involved in human or canine aspergillosis. Interestingly, in cats infection occurs in apparently systemically immunocompetent individuals, whereas human and canine infections have occurred in immunocompromised patients.[2] Around 40% of affected cats are pure-breeds of Persian lineage and have a brachycephalic facial conformation with foreshortening of the skull and anterior rotation of the nasal bones.[70] Infection may be more likely in cats due to an environmental exposure factor (e.g., their exposure to fungal spores in the environment may be secondary to a particular feline behaviour such as burying faeces) and/or genetic factors such as a deficient innate immune response (e.g., problems with local immune factors).[78]

Antifungal therapy

The current recommended primary therapy for invasive aspergillosis in humans is voriconazole, with isavuconazole and amphotericin B acceptable as alternatives for treatment failures.[79] Given AVC species can have high MICs of voriconazole,[4,21–26] patient management can be greatly impacted by the failure to recognise the aetiological agent, leading to treatment failure and protracted illness. Therefore, correct identification of the species causing invasive aspergillosis is crucial for optimal patient treatment.

The optimal treatment for cats with SOA caused by *A. viridinutans* complex species is yet to be identified. Most treated cats have been euthanased due to disease progression despite aggressive antifungal therapy using itraconazole or posaconazole as monotherapy or combined with amphotericin B, or with amphotericin B and terbinafine.[71] A benefit of adding exenteration to the treatment regime has not been demonstrated. Voriconazole can have serious adverse effects on cats and cannot be used for first line therapy.[71,80,81] The echinocandin caspofungin was effective in one case that failed therapy with triazoles and amphotericin B.[71]

Antifungal resistance

Azole resistance amongst *Aspergillus species* is an increasing global problem and can be primary (inherent) or secondary (acquired). Mutations in the *cyp51A* gene are associated with secondary azole resistance amongst human clinical isolates of *A. fumigatus*.[82–85] *Cyp51A* mutations have not yet been investigated in AVC species. The draft genome sequence of *A. udagawae* has been recently released and may help identify regions that contribute to resistance mechanisms.[86]

Resistance mechanisms have been investigated for another cryptic *A.* section *Fumigati* species, *A. lentulus*.[87,88] These studies concluded that the *cyp51A* mechanisms conferring resistance for *A. lentulus* differ from that of *A. fumigatus.* A three-dimensional protein model was used to explore interactions between *cyp51A* and voriconazole.[88] When *A. fumigatus* and *A. lentulus* *cyp51A* proteins were compared, a difference in the BC loop of the protein led to differences in the BC loop lock-up of voriconazole, which could account for differences in antifungal susceptibility profiles between these two species. The prevalence of acquired versus inherent resistance mechanisms amongst AVC species is unknown. Epidemiological cut-off values (ECVs) for antifungal susceptibility testing have not yet been established for this complex. The MICs of all classes of antifungal drugs are often higher in AVC species compared to ECVs established for *A. fumigatus* (Table 3).[89–91]*In vitro* and *in vivo* resistance to triazoles in human patients with AVC infections, have resulted in treatment failure.[6,11,21,23,26,27] One patient with chronic invasive pulmonary aspergillosis due to *A. parafelis* infection failed to respond to itraconazole initially, had a partial response to posaconazole, then relapsed during voriconazole therapy.[4,27] Combination therapy with posaconazole and caspofungin was also unsuccessful, and the patient died. Antifungal susceptibility testing of the infecting *A. parafelis* isolate obtained at post mortem demonstrated high MIC values of voriconazole (4 μg/ml) and itraconazole (>16 μg/ml).[4,27]

Another patient with leukaemia and invasive pulmonary aspergillosis due to *A. felis* was treated using combination therapy with voriconazole, caspofungin and amphotericin B. The patient had a mixed infection with other *Aspergillus* sp. (initially *A. calidoustus* and *A. novofumigatus*, then *A. novofumigatus* and *A. felis*) and died. All infecting isolates had high MICs of voriconazole (4–8 μg/ml).[24–26]

High MICs of amphotericin B, itraconazole and voriconazole have been observed among *A. udagawae* isolates from clinical infections when compared to *A. fumigatus*, via microbroth

dilution methods.[21] Clinically, infections were also refractory to therapy, with longer infection duration observed among chronic granulomatous disease (CGD) patients with IA due to *A. udagawae* (mean duration 35 weeks) compared to CGD patients with IA due to *A. fumigatus* (mean duration 5.5 weeks).[21] High MICs of amphotericin B and voriconazole have also been observed in other reports of invasive infection due to *A. udagawae*.[6] In an ocular *A. udagawae* infection, therapy with itraconazole tablets and ophthalmic drops failed to clear the infection and the eye was exenterated. The isolate had a high MIC of voriconazole (8 μg/ml) but appeared to be susceptible to itraconazole based on a low MIC value (0.25 μg/ml).[23]

High MICs of itraconazole, voriconazole, and ravuconazole and susceptibility to amphotericin B and echinocandins were reported for two clinical isolates of *A. viridinutans*[11] that were later revealed to be *A. pseudofelis* (NRRL 62902) and *A. parafelis* (NRRL 62900).[4]

Other cases of human infection due to *A. udagawae* that were treated successfully with triazoles had high MIC values of nonazole antifungals. In One case of bronchial aspergillosis, decreased susceptibility to amphotericin B compared to *A. fumigatus sensu stricto* was observed.[22] In a patient with *A. udagawae* infection causing ARDS, treatment with amphotericin B and methylprednisone was unsuccessful, although no antifungal susceptibility testing was performed in this case.[75]

The extended spectrum triazole isavuconazole has been tested against one environmental and nine clinical isolates of *A. udagawae*.[92] Many of the isolates demonstrated decreased susceptibility to itraconazole (Mode MIC 1 μg/ml, geometric mean 0.660 μg/ml) and voriconazole (Mode MIC of 1 μg/ml, geometric mean 0.812 μg/ml) but increased susceptibility to isavuconazole (Mode MIC 0.125 μg/ml, geometric mean 0.100 μg/ml). This drug shows promise for clinical treatment of invasive aspergillosis due to *A. udagawae*. The susceptibility of other species in the AVC to isavuconazole is yet to be determined.

Antifungal susceptibilities of *A. felis* isolates from cats have been reported, with generally low MICs for amphotericin B and echinocandins. Some isolates had high MICs of triazoles, and cross-resistance to triazoles was also observed.[4,24] Results of antifungal susceptibility testing should inform antifungal selection in treatment of individual cats. Infecting isolates can be readily cultured from clinical biopsies of sinonasal mucosa or orbital masses on commercial media, for example, Sabouraud's dextrose agar or malt extract agar at 37°C.[71]

The *Aspergillus viridinutans* complex contains many clinically relevant species and recently discovered species of unknown pathogenicity. Known pathogenic species in the complex can affect both human and animal patients, reflecting the scope for a One-Health approach to disease investigation. Human and veterinaryclinicians should be aware of the emerging threat members of this complex pose to susceptible patients. Knowledge of their epidemiology is important for exposure risk assessment and differential diagnoses. Their seemingly global distribution further highlights the need for clinician awareness. Members of this complex share some morphological and physiological characteristics and relatedness is confirmed by phylogeny based on combined multi-locus sequence analysis. Given that members of the *A. viridinutans* complex have seemingly inherent resistance to commonly used triazole antifungal therapeutics and infections usually have a poor to grave clinical prognosis, it is important to correctly identify agents of disease through polyphasic taxonomy and to perform antifungal susceptibility testing. This will assist both human and veterinary practitioners with prognosis assessment and case management.

Acknowledgements

The authors wish to thank Dr Jos Houbraken of the Westerdijk Fungal Biodiversity Institute for technical assistance with fungal light microscopy imaging.

Publication Figures

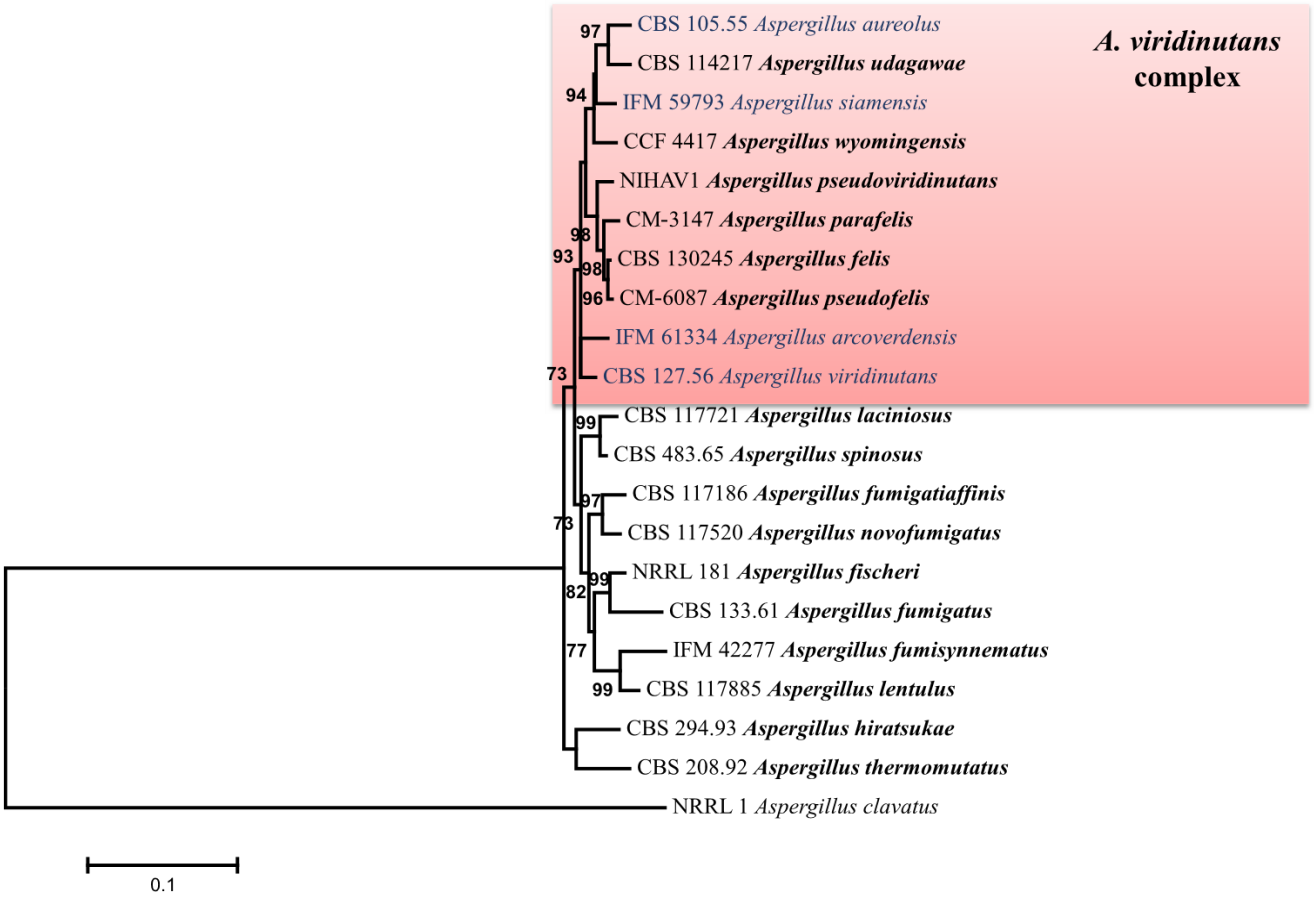


Figure 1. Phylogenetic tree depicting species in the *Aspergillus viridinutans* complex and pathogenic species in section *Fumigati* based on *BenA* and *CaM* DNA sequences of type strains.[4,54] All pathogenic species are set in bold. Nonpathogenic species are in blue font. Three pathogenic species in section *Fumigati: A. beijingensis, A. qizutongii,* and *A. wangduanlii* were not included since *BenA* and *CaM* sequence data are not available. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model[94] with 1,000 bootstrap replicates.[95] The tree with the highest log likelihood (−3539.8604) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches for values >70%. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories +G, parameter = 0.8578). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6.[96]

Figure 2. Nodding conidial head typical of *Aspergillus viridinutans* complex species. *A. parafelis* isolate ×100 light microscopy image.

Publication tables

Table 1. Environmental *Aspergillus viridinutans* complex isolates.

Species	Sources	Geographic region	Publications
A. arcoverdensis (*n* = 11)	Semi-desert soil	Brazil (*n* = 11)	Matsuzawa et al. 2015[35]
A. aureolus (*n* = 6)	Soil	Ghana (*n* = 2), Liberia (*n* = 1), Brazil (*n* = 2), unknown (*n* = 1)	Fennell and Raper 1955[32]; Raper and Fennell 1965[33]; Horie et al. 1995[34]; Nováková et al. 2014[31]
A. felis (*n* = 12)	Indoor air Soil Food	Germany (*n* = 1), USA (*n* = 4), Sri Lanka (n = 2), India (*n* = 1), Czech Republic (*n* = 1), Japan (*n* = 1),	Barrs et al. 2013[24]; Nováková et al. 2014[31]; Gonçalves et al. 2016[55]
	Pinus caribea	Chile (n = 1), Zambia (n = 1)	
	Rocks		
A. siamensis (*n* = 3)	Coastal forest soil	Thailand (*n* = 3)	Eamvijarn et al. 2013[36]
A. udagawae (*n* = 57)	Soil Rocks	USA (*n* = 53), Brazil (*n* = 3) Chile (*n* = 1)	Hong et al. 2010[93]; Horie et al. 1995[34]; Nováková et al. 2014[31]; Gonçalves et al. 2016[55]
A. viridinutans sensu stricto (n = 3)	Rabbit dung Sandy podzol Soil	Australia (*n* = 3)	McLennan et al. 1954[39]; Katz et al. 2005[28,a]
A. wyomingensis (n = 14)	Soil	USA (*n* = 12) China (*n* = 1) Russia (*n* = 1)	Nováková et al. 2014[31]; Varga et al. 2000[66]

[a] Identification questionable: nucleotide homology < 99% with type strain sequence.

Table 2. Published clinical cases caused by *Aspergillus viridinutans* complex species where molecular sequencing techniques were used in addition to morphological identification.[a]

Fungal species	Host	Origin	Source	Publication	Gene sequenced
A. felis (*n* = 14)	Cat	Australia	Retrobulbar mass	Barrs et al. 2013[24]	*ITS, BenA, CaM*
				Katz et al. 2005[28]	*Alkaline protease, BenA, ITS*
A. felis (*n* = 1)	Dog	Australia	Vitreous humor	Barrs et al. 2013[24]	*ITS, BenA, CaM*
A. felis (*n* = 1)	Cat	UK	Retrobulbar mass	Barrs et al. 2013[24]	*ITS, BenA, CaM*
A. felis (*n* = 1)	Cat	Australia	Thoracic mass	Barrs et al. 2013[24]	*ITS, BenA, CaM*
A. felis (*n* = 1)	Cat	Australia	Sino-nasal cavity	Barrs et al. 2013[24]	*ITS, BenA, CaM*
A. felis (*n* = 1)	Human	Spain	Sputum, BAL	Barrs et al. 2013[24]	*ITS, BenA, CaM*
				Peláez et al. 2013[26]	
A. felis (*n* = 1)	Human	Portugal	Lung	Barrs et al. 2013[24]Coelho et al. 2011[27]	*ITS, BenA, CaM, rodA*
A. felis (*n* = 1)	Human	Japan	Thigh bone abscess	Novakova et al. 2014[88]	*BenA, CaM*
A. parafelis (*n* = 1)	Human	Portugal	Lung	Sugui et al. 2014[4]	*BenA, CaM, MCM7, RPB2, TSR1*

A. parafelis (*n* = 1)	Human	Spain	Oropharyngeal exudate	Sugui et al. 2014[4]Alcazar-Fuoli et al. 2008[11]	*BenA, CaM, MCM7, RPB2, TSR1, rodA*
A. pseudofelis (*n* = 1)	Human	Spain	Nail	Sugui et al. 2014[4]Alcazar-Fuoli et al. 2008[11]	*BenA, CaM, MCM7, RPB2, TSR1, rodA*
A. pseudofelis (*n* = 1)	Human	Spain	Sputum	Sugui et al. 2014[4]	*BenA, CaM, MCM7, RPB2, TSR1*
A. pseudoviridinutans (*n* = 1)	Human	USA	Lymph node	Sugui et al. 2014[4]Vinh et al. 2009[29]	*BenA, CaM, MCM7, RPB2, TSR1*
A. udagawae (*n* = 1)	Human	Japan	BAL fluid	Gyotoku et al. 2012[22]	*BenA*[a]
A. udagawae (*n* = 1)	Human	USA	BAL fluid	Farrell et al. 2014[75]	*ITS, BenA, CaM*
A. udagawae (*n* = 1)	Human	Italy	Cornea	Posteraro et al. 2011[23]	*ITS, BenA, CaM*
A. udagawae (*n* = 4)	Human	USA	Lung	Vinh et al. 2009[21]	*ITS, BenA, rodA*
A. udagawae (*n* = 1)	Human	Czech Republic	Nail	Novakova et al. 2014[31]	*CaM*[a]
A. udagawae (*n* = 2)	Human	USA	Lung	Sugui et al. 2010[76]	*BenA, rodA*
A. udagawae (*n* = 3)	Human	Japan	Unknown	Tamiya et al. 2015[61]	*BenA, CaM*
A. udagawae (*n* = 1)	Human	Japan	BAL fluid	Tamiya et al. 2015[61]	*BenA, CaM*
A. udagawae (*n* = 1)	Human	Japan	Ocular bulb	Tamiya et al. 2015[61]	*BenA, CaM*
A. udagawae (*n* = 3)	Human	USA	BAL fluid	Balajee et al. 2006[6]	*BenA, rodA, RFLP*[a]
A. udagawae (*n* = 1)	Human	USA	Lung	Balajee et al. 2006[6]	*BenA, rodA, RFLP*[a]
A. udagawae (*n* = 2)	Human	Unknown	Unknown	Balajee et al. 2006[6]	*BenA, rodA, RFLP*[a]

A. udagawae (*n* = 1)	Human	Unknown	Brain	Balajee et al. 2006[6]	*BenA, rodA, RFLP*[a]
A. udagawae (*n* = 2)	Human	Spain	Unknown	Escribano et al. 2013[14]	*BenA*[a]
A. udagawae (*n* = 1)	Cat	Australia	Sinonasal cavity	Barrs et al. 2014[2]	*BenA, CaM*
A. udagawae (*n* = 1)	Cat	Australia	Respiratory tract	Barrs et al. 2013[24] Katz et al. 2005[28]	*ITS, BenA, CaMAlkaline protease, BenA, ITS*
A. udagawae (*n* = 1)	Cat	USA	Retrobulbar mass	Barrs et al. 2014[2]	*CaM*[a]
A. udagawae (*n* = 2)	Cat	Japan	Retrobulbar mass	Kano et al. 2008, 2013[72,73]	*ITS, BenA*[a]
A. udagawae (*n* = 3)	Human	USA	Lung	Balajee et al. 2009[8]	*ITS, BenA*[a]
A. viridinutans (*n* = 1)	Human	Japan	Cornea	Shigeyasu 2012[30]	*BenA*[a]
A. viridinutans (*n* = 1)	Human	Japan	Unknown	Tamiya et al. 2015[61]	*BenA, CaM*[a]
A. viridinutans (*n* = 1)	Human	Japan	Lung	Tamiya et al. 2015[61]	*BenA, CaM*[a]
A. viridinutans (*n* = 1)	Human	USA	Lung	Vinh et al. 2009[29]	*ITS, BenA, rodA*[a]
A. viridinutans (*n* = 1)	Human	Spain	Unknown	Alastruey-Izquierdo et al. 2013[64]	*ITS, BenA*[a]
A. viridinutans (*n* = 1)	Human	Spain	Unknown	Escribano et al. 2013[14]	*BenA*[a]
A. viridinutans (*n* = 1)	Human	Norway	Brain	Andersen et al. 2012[77]	*ITS*[a]
A. wyomingensis (*n* = 1)	Cat	Australia	Retrobulbar mass	Barrs et al. 2014[2]	*ITS, BenA*

[a] Identification questionable: nucleotide homology <99% with type strain sequence, no GenBank sequence available or only one gene sequenced.

BAL = Bronchoalveolar lavage.

Table 3. Antifungal susceptibility profiles reported for clinical isolates of *Aspergillus viridinutans* complex species and one environmental isolate (*A. viridinutans*) based on CLSI M38-A2,[4,11,21–23,26,27] EUCAST and Sensititre YO10 broth microdilution methods.[24]

Drug (ECV µg/ml for wild-type *A. fumigatus sensu stricto*)	**Species**	**Number of isolates tested**	**MIC/MEC range (µg/ml)**	**MIC/MEC Geometric Mean (µg/ml)**
Amphotericin B* (2)[89]	*A. felis*[4,24,26]	15	0.25–1	0.55
	A. parafelis[4,11,27]	5	0.37–2	0.99
	A. pseudofelis[4]	2	2	2.00
	A. pseudoviridinutans[4]	2	2	2.00
	A. udagawae[6,21–23]	14	0.125–4	1.53
	A. viridinutans[4]	1	0.25	0.25
Itraconazole* (0.25)[90]	*A. felis*[4,24,26]	15	0.03–8	0.31
	A. parafelis[4]	5	14.4–>16	15.17
	A. pseudofelis[4]	2	>16	>16.00
	A. pseudoviridinutans[4]	2	2–>16	5.65
	A. udagawae[6,21–23]	14	0.125–4	0.48
	A. viridinutans[4]	1	1	1.00
Voriconazole* (1)[90]	*A. felis*[4,24,26]	15	0.25–8	1.45
	A. parafelis[4]	5	4–8	5.28
	A. pseudofelis[4]	2	8	8.00
	A. pseudoviridinutans[4]	2	2–8	4.00
	A. udagawae[6,21–23]	14	0.25–>16	1.12
	A. viridinutans[4]	1	1	1.00
Posaconazole* (0.25)[90]	*A. felis*[24,26]	14	0.03–4	0.14
	A. parafelis[4]	3	0.25–0.41	0.29
	A. udagawae[21,23]	5	0.25–0.5	0.33

Terbinafine*	*A. felis*[24]	13	0.25	0.25
	A. parafelis[4]	2	0.75–1.2	0.95
	A. udagawae[21]	4	0.25–1	0.40
Caspofungin† (1)[91]	*A. felis*[24,26]	14	0.008–2	0.06
	A. parafelis[4]	3	<0.016–1	0.82
	A. udagawae[6,21,23]	12	0.015–0.5	0.05
Anidulafungin†	*A. felis*[24,26]	14	0.015–<0.03	0.02
	A. parafelis[4]	1	<0.016	<0.02
	A. udagawae[23]	1	0.016	0.02
Micafungin†	*A. felis*[24,26]	14	0.008–<0.03	0.01
	A. parafelis[4]	2	0.03–2	0.07
	A. udagawae[21–23]	6	<0.015–0.25	0.25
Fluconazole*	*A. udagawae*[23]	1	>256	>256.00

*MIC, minimum inhibitory concentration; †MEC, minimum effective concentration.

Publication References

1. Frisvad JC, Larsen TO. Extrolites of *Aspergillus fumigatus* and other pathogenic species in *Aspergillus* section *Fumigati*. *Front Microbiol*. 2016; **6**: 1–14.

2. Barrs VR, Beatty JA, Dhand NK et al. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. *Vet J*. 2014; **201**: 215–222.

3. Howard SJ. Multi-resistant aspergillosis due to cryptic species. *Mycopathologia*. 2014; **178**: 435–439.

4. Sugui JA, Peterson SW, Figat A et al. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J Clin Microbiol*. 2014; **52**: 3707–3721.

5. Balajee SA, Gribskov JL, Hanley E et al. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. *Eukaryot Cell*. 2005; **4**: 625–632.

6. Balajee SA, Nickle D, Varga J et al. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryot Cell*. 2006; **5**: 1705–1712.

7. Zbinden A, Imhof A, Wilhelm MJ et al. Fatal outcome after heart transplantation caused by *Aspergillus lentulus*. *Transpl Infect Dis*. 2012; **14**: E60–E63.

8. Balajee SA, Kano R, Baddley JW et al. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *J Clin Microbiol*. 2009; **47**: 3138–3141.

9. Montenegro G, Sánchez Puch S, Jewtuchowicz VM et al. Phenotypic and genotypic characterization of *Aspergillus lentulus* and *Aspergillus fumigatus* isolates in a patient with probable invasive aspergillosis. *J Med Microbiol*. 2009; **58** (Pt 3): 391–395.

10. Alhambra A, Catalán M, Moragues MD et al. Isolation of *Aspergillus lentulus* in Spain from a critically ill patient with chronic obstructive pulmonary disease. *Rev Iberoam Micol*. 2008; **25**: 246–249.

11. Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A et al. *Aspergillus* Section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. *Antimicrob Agents Chemother*. 2008; **52**: 1244–1251.

12. Kidd SE, Goeman E, Meis JF et al. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses*. 2015; **58**: 350–355.

13. Sabino R, Veríssimo C, Parada H et al. Molecular screening of246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. *Med Mycol*. 2014; **52**: 519–529.

14. Escribano P, Peláez T, Munoz P et al. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? *Antimicrob Agents Chemother*. 2013; **57**: 2815–2820.

15. Yoshida H, Seki M, Umeyama T et al. Invasive pulmonary aspergillosis due to *Aspergillus lentulus*: Successful treatment of a liver transplant patient. *J Infect Chemother*. 2015; **21**: 479–481.

16. Mortensen KL, Johansen HK, Fuursted K et al. A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. *Eur J Clin Microbiol Infect Dis*. 2011; **30**: 1355–1363.

17. Symoens F, Haase G, Pihet M et al. Unusual *Aspergillus* species in patients with cystic fibrosis. *Med Mycol*. 2010; **48** (S1): S10–S16.

18. de Azevedo Bastos VR, de Castro Lima Santos DW, Padovan ACB et al. Early invasive pulmonary aspergillosis in a kidney transplant recipient caused by *Aspergillus lentulus*: first Brazilian report. *Mycopathologia*. 2015; **179**: 299–305.

19. Negri CE, Goncalves SS, Xafranski H et al. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. *J Clin Microbiol*. 2014; **52**: 3633–3640.

20. Balajee SA, Gribskov J, Brandt M et al. Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. *J Clin Microbiol*. 2005; **43**: 5996–5999.

21. Vinh DC, Shea YR, Sugui JA et al. Invasive aspergillosis due to *Neosartorya udagawae*. *Clin Infect Dis.* 2009; **49**: 102–111.

22. Gyotoku H, Izumikawa K, Ikeda H et al. A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. *Med Mycol*. 2012; **50**: 631–636.

23. Posteraro B, Mattei R, Trivella F et al. Uncommon *Neosartorya udagawae* fungus as a causative agent of severe corneal infection. *J Clin Microbiol*. 2011; **49**: 2357–2360.

24. Barrs VR, van Doorn TM, Houbraken J et al. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One*. 2013; **8**: e64871.

25. Alvarez-Pérez S, Mellado E, Serrano D et al. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. *J Med Microbiol*. 2014; **63** (Pt 4): 617–619.

26. Peláez T, Alvarez-Pérez S, Mellado E et al. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. *JMed Microbiol*. 2013; **62** (Pt 3): 474–478.

27. Coelho D, Silva S, Vale-Silva L et al. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. *Med Mycol.* 2011; **49**: 755–759.

28. Katz ME, Dougall AM, Weeks K et al. Multiple genetically distinct groups revealed among clinical isolates identified as atypical *Aspergillus fumigatus*. *J Clin Microbiol.* 2005; **43**: 551–555.

29. Vinh DC, Shea YR, Jones PA et al. Chronic invasive aspergillosis caused by *Aspergillus viridinutans*. *Emerg Infect Dis.* 2009; **15**: 1292–1294.

30. Shigeyasu C, Yamada M, Nakamura N et al. Keratomycosis caused by *Aspergillus viridinutans*: an *Aspergillus fumigatus* resembling mold presenting distinct clinical and antifungal susceptibility patterns. *Med Mycol*. 2012; **50**: 525–528.

31. Nováková A, Hubka V, Dudová Z et al. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (USA) and revision of *A. viridinutans* complex. *Fungal Diversity*. 2014; **64**: 253–274.

32. Fennell DI, Raper KB. New species and varieties of *Aspergillus*. Mycologia. 1955; **47**: 71–75.

33. Raper KB, Fennell DI. *The Genus Aspergillus*. Baltimore: Williams and Wilkins; 1965.

34. Horie Y, Miyaji M, Nishimura K et al. New and interesting species of *Neosartorya* from Brazilian soil. *Mycoscience*. 1995; **36**: 199–204.

35. Matsuzawa T, Takaki GMC, Yaguchi T et al. *Aspergillus arcoverdensis*, a new species of *Aspergillus* section *Fumigati* isolated from caatinga soil in State of Pernambuco, Brazil. *Mycoscience*. 2015; **56**: 123–131.

36. Eamvijarn A, Manoch L, Chamswarng C et al. *Aspergillus siamensis* sp. nov. from soil in Thailand. *Mycoscience*. 2013; **54**: 401–405.

37. Samson RA, Hong S, Peterson SW et al. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Stud Mycol*. 2007; **59**: 147–203.

38. Samson RA, Visagie CM, Houbraken J et al. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud Mycol.* 2014; **78**: 141–173.

39. McLennan EI, Ducker SC, Thrower LB. New soil fungi from Australian heathland: *Aspergillus, Penicillium, Spegazzinia*. *Aust J Bot.* 1954; **2**: 355–364.

40. Subramanian CV. The perfect states of *Aspergillus. Curr Sci India.* 1972; **41**: 755–761.

41. Malloch D, Cain RF. The *Trichocomataceae*: Ascomycetes with *Aspergillus, Paecilomyces* and *Penicillium* imperfect states. *Can J Bot*. 1972; **50**: 2613–2628.

42. Samson RA, WG. *Aspergillus aureoluteus*. In: Samson RA, Pitt JI, eds. *Advances in Penicillium and Aspergillus Systematics*. New York: Springer Science+Business Media, 1985, 31–54.

43. Hong SB, Go SJ, Shin HD et al. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia*. 2005; **97**: 1316–1329.

44. McNeill J, Barrie FR, Buck WR et al. International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) adopted by the Eighteenth International Botanical Congress Melbourne, Australia, July 2011. *Konigstein: Koeltz Scientific Books*. 2012.

45. White TJ, Bruns T, Lee SJWT. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York: Academic Press, 1990, 315–322.

46. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol*. 1995; **61**: 1323–1330.

47. Schmitt I, Crespo A, Divakar PK et al. New primers for promising single-copy genes in fungal phylogenetics and systematics. *Persoonia*. 2009; **23**: 35–40.

48. Liu YJ, Whelen S, Hall BD. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Mol Biol Evol*. 1999; **16**: 1799–1808.

49. Peterson SW. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia*. 2008; **100**: 205–226.

50. Lillehoj EB, Milburn MS. Viriditoxin production by *Aspergillus viridi-nutans* and related species. *Appl Microbiol*. 1973; **26**: 202–205.

51. Lillehoj EB, Ciegler A. A toxic substance from *Aspergillus viridinutans*. *Can J Microbiol*. 1972; **18**: 193–197.

52. Hong SB, Shin HD, Hong J et al. New taxa of *Neosartorya* and *Aspergillus* in *Aspergillus* section *Fumigati*. *Antonie Van Leeuwenhoek*. 2008; **93**: 87–98.

53. Weisleder D, Lillehoj EB. Structure of viriditoxin, a toxic metabolite of *Aspergillus viridinutans*. *Tetrahedron Lett.* 1971; **48**: 4705–4706.

54. Cole RJ, Cox RH. *Handbook of Toxic Fungal Metabolites*. New York: Academic Press; 1981.

55. Goncalves VN, Cantrell CL, Wedge DE et al. Fungi associated with rocks of the Atacama Desert: taxonomy, distribution, diversity, ecology and bioprospection for bioactive compounds. *Environ Microbiol*. 2016; **18**: 232–245.

56. Ramos AA, Prata-Sena M, Castro-Carvalho B et al. Potential of four marine-derived fungi extracts as anti-proliferative and cell death-inducing agents in seven human cancer cell lines. *Asian Pac J Trop Med*. 2015; **8**: 798–806.

57. Buttachon S, Chandrapatya A, Manoch L et al. Sartorymensin, a new indole alkaloid, and new analogues of tryptoquivaline and fiscalins produced by *Neosartorya siamensis* (KUFC 6349). *Tetrahedron*. 2012; **68**: 3253–3262.

58. Mendes G, Gonc¸alves VN, Souza-Fagundes EM et al. Antifungal activity of extracts from Atacama Desert fungi against *Paracoccidioides brasiliensis* and identification of *Aspergillus felis* as a promising source of natural bioactive compounds. *Mem Inst Oswaldo Cruz*. 2016; **111**: 209–217.

59. Xiao L, Liu H, Wu N et al. Characterization of the high cytochalasin E and rosellichalasin producing-*Aspergillus* sp. nov. F1 isolated from marine solar saltern in China. *World J Microbiol Biotechnol*. 2013; **29**: 11–17.

60. Naruse N, Yamamoto H, Murata S et al. Aspochalasin E, a new antibiotic isolated from a fungus. *J Antibiot (Tokyo)*. 1993; **46**: 679–681.

61. Tamiya H, Ochiai E, Kikuchi K et al. Secondary metabolite profiles and antifungal drug susceptibility of *Aspergillus fumigatus* and closely related species, *Aspergillus lentulus, Aspergillus udagawae,* and *Aspergillus viridinutans*. *J Infect Chemother*. 2015; **21**: 385–391.

62. National Center for Biotechnology Information. Basic Local Alignment Search Tool (BLAST): Standard Nucleotide BLAST. https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed 2016.

63. Verwer PEB, van Leeuwen WB, Girard V et al. Discrimination of *Aspergillus lentulus* from *Aspergillus fumigatus* by Raman spectroscopy and MALDI-TOF MS. *Eur J Clin Microbiol Infect Dis*. 2014; **33**: 245–251.

64. Alastruey-Izquierdo A, Mellado E, Peláez T et al. Population based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrob Agents Chemother*. 2013; **57**: 3380–3387.

65. Gautier M, Normand AC, Ranque S. Previously unknown species of *Aspergillus*. *Clin Microbiol Infect*. 2016; **S1198-743X**: 30140–30149.

66. Varga J, Toth B, Rigo K et al. Genetic variability within the *Aspergillus viridinutans* species. *Folia Microbiol (Praha)*. 2000; **45**: 423–428.

67. Staab JF, Balajee SA, Marr KA. *Aspergillus* section *Fumigati* typing by PCR-restriction fragment polymorphism. *J Clin Microbiol*. 2009; **47**: 2079–2083.

68. Adhikari M, Kim S, Yadav DR et al. A new record of *Neosartorya aureola* isolated from field soil in Korea. *Korean J Mycol*. 2015; **43**: 191–195.

69. Talbot JJ, Johnson LR, Martin P et al. What causes canine sinonasal aspergillosis? A molecular approach to species identification. *Vet J*. 2014; **200**: 17–21.

70. Barrs VR, Talbot JJ. Feline aspergillosis. *Vet Clin North Am Small Anim Pract*. 2014; **44**: 51–73.

71. Barrs VR, Halliday C, Martin P et al. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *Vet J*. 2012; **191**: 58–64.

72. Kano R, Itamoto K, Okuda M et al. Isolation of *Aspergillus udagawae* from a fatal case of feline orbital aspergillosis. *Mycoses*. 2008; **51**: 360–361.

73. Kano R, Shibahashi A, Fujino Y et al. Two cases of feline orbital aspergillosis due to *Aspergillus udagawae* and *A. viridinutans*. *J Vet Med Sci.* 2013; **75**: 7–10.

74. Tamborini A, Robertson E, Talbot JJ et al. Sinonasal aspergillosis in a British Shorthair cat in the UK. *J Feline Med Surg Open Reports*. 2016; **2**: 1–6.

75. Farrell JJ, Kasper DJ, Taneja D et al. Acute respiratory distress caused by *Neosartorya udagawae*. *Med Mycol Case Rep*. 2014; **24**: 1–5.

76. Sugui JA, Vinh DC, Nardone G et al. *Neosartorya udagawae* (*Aspergillus udagawae*), an emerging agent of aspergillosis: how different is it from *Aspergillus fumigatus*? *J Clin Microbiol*. 2010; **48**: 220–228.

77. Andersen CT, Bechensteen AG, Hesstvedt L et al. Multiple brain abscesses caused by *Aspergillus viridinutans* in a leukemic child. *Mycoses*. 2012; **55** (S4): 75.

78. Goncalves SM, Lagrou K, Duarte-Oliveira C et al. The microbiome-metabolome crosstalk in the pathogenesis of respiratory fungal diseases. *Virulence*. 2016; Nov 7 epub ahead of print http://dx.doi.org/10.1080/21505594.2016.1257458.

79. Patterson TF, Thompson GR, III, Denning DW et al. Practical guidelines for the diagnosis and management of aspergillosis: 2016 update by the infectious diseases society of America. *Clin Infect Dis*. 2016; **63**: e1–e60.

80. Quimby JM, Hoffman SB, Duke J et al. Adverse neurologic events associated with voriconazole use in 3 cats. *J Vet Intern Med*. 2010; **24**: 647–649.

81. Smith LN, Hoffman SB. A case series of unilateral orbital aspergillosis in three cats and treatment with voriconazole. *Vet Opthalmol*. 2010; **13**: 190–203.

82. van der Linden JW, Warris A, Verweij PE. *Aspergillus* species intrinsically resistant to antifungal agents. *Med Mycol*. 2011; **49** (S1): S82–S89.

83. Howard SJ, Arendrup MC. Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection. *Med Mycol*. 2011; **49** (S1): S90–S95.

84. Chen J, Li H, Li R et al. Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *J Antimicrob Chemother* 2005; **55**: 31–37.

85. Verweij PE, Kema GH, Zwaan B et al. Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould *Aspergillus fumigatus*. *Pest Manag Sci.* 2013; **69**: 165–170.

86. Kusuya Y, Takahashi-Nakaguchi A, Takahashi H et al. Draft genome sequence of the pathogenic filamentous fungus *Aspergillus udagawae* strain IFM 46973T. *Genome Announc*. 2015; **3**: e00834–15.

87. Mellado E, Alcazar-Fuoli L, Cuenca-Estrella M et al. Role of *Aspergillus lentulus* 14-*α* sterol demethylase (*Cyp51A*) in azole drug susceptibility. *Antimicrob Agents Chemother*. 2011; **55**: 5459–5468.

88. Alcazar-Fuoli L, Cuesta I, Rodriguez-Tudela JL et al. Three dimensional models of 14*αα*-sterol demethylase (*Cyp51A*) from *Aspergillus lentulus* and *Aspergillus fumigatus*: an insight into differences in voriconazole interaction. *Int J Antimicrob Agents*. 2011; **38**: 426–434.

89. Espinel-Ingroff A, Cuenca-Estrella M, Fothergill A et al. Wildtype MIC distributions and epidemiological cut off values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Antimicrob Agents Chemother*. 2011; **55**: 5150–5154.

90. Espinel-Ingroff A,DiekemaDJ, Fothergill A et al. Wild-type MIC distributions and epidemiological cut off values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 Document). *J Clin Microbiol*. 2010; **48**: 3251–3257.

91. Espinel-Ingroff A, Fothergill A, Fuller J et al. Wild-type MIC distributions and epidemiological cut off values for caspofungin and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 Document). *Antimicrob Agents Chemother*. 2011; **55**: 2855–2859.

92. Datta K, Rhee P, Byrnes E, III et al. Isavuconazole activity against *Aspergillus lentulus*, *Neosartorya udagawae*, and *Cryptococcus gattii*, emerging fungal pathogens with reduced azole susceptibility. *J Clin Microbiol*. 2013; **51**: 3090–3093.

93. Hong SB, Kim DH, Park IC et al. Re-identification of *Aspergillus fumigatus* sensu lato based on a new concept of species delimitation. *J Microbiol*. 2010; **48**: 607–615.

94. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol*. 1980; **16**: 111–120.

95. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985; **39**: 783–791.

96. Tamura K, Stecher G, Peterson D et al.MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*. 2013; **30**:2725–2729.

2.2.3 Conclusions

The AVSC is a medically important group of cryptic fungi found in a variety of environments. Although not all member species have been identified as pathogenic, continued surveillance for these cryptic species amongst clinical isolates is warranted to identify patients at higher risk of treatment failure and to monitor pathogenicity within the complex.

Since this review, the antifungal susceptibility data of a large group of AVSC isolates have been published (Lyskova et al., 2018). The recent study, published in February of 2018, compared the *in vitro* microbroth dilution antifungal susceptibility methods of EUCAST (EUCAST, 2017) and Sensititre™ YeastOne™ (Thermo Scientific Trek Diagnostic Systems, USA on environmental and clinical AVSC isolates (n=90) for triazoles (voriconazole, posaconazole, itraconazole), amphotericin B and echinocandins (anidulafungin, micafungin and caspofungin). The study included isolates of *A. udagawae, A. felis, A. wyomingensis, A. arcoverdensis, A. pseudoviridinutans, A. aureolus, A. siamensis, A. viridinutans*, as well as isolates from a suspected new species, temporarily called *A. acrensis* (Lyskova et al., 2018) (the species description for *A. acrensis* sp. nov. was recently accepted for publication (Hubka et al., 2018)). EUCAST testing returned low MICs of posaconazole and MECs of echinocandins for most AVSC isolates, and compared to EUCAST testing, the Sensititre™ YeastOne™ method could not detect elevated MICs of itraconazole in AVSC isolates. Overall the study found an agreement between the two *in vitro* methods of 67% (when Sensititre™ YeastOne™ results were read at 48 hours) to 82% (when read at 72 hours) (Lyskova et al., 2018).

2.3 Azole resistance in *Aspergillus fumigatus* and the *cyp51A* gene

2.3.1 Triazole antifungals and the *cyp51* gene

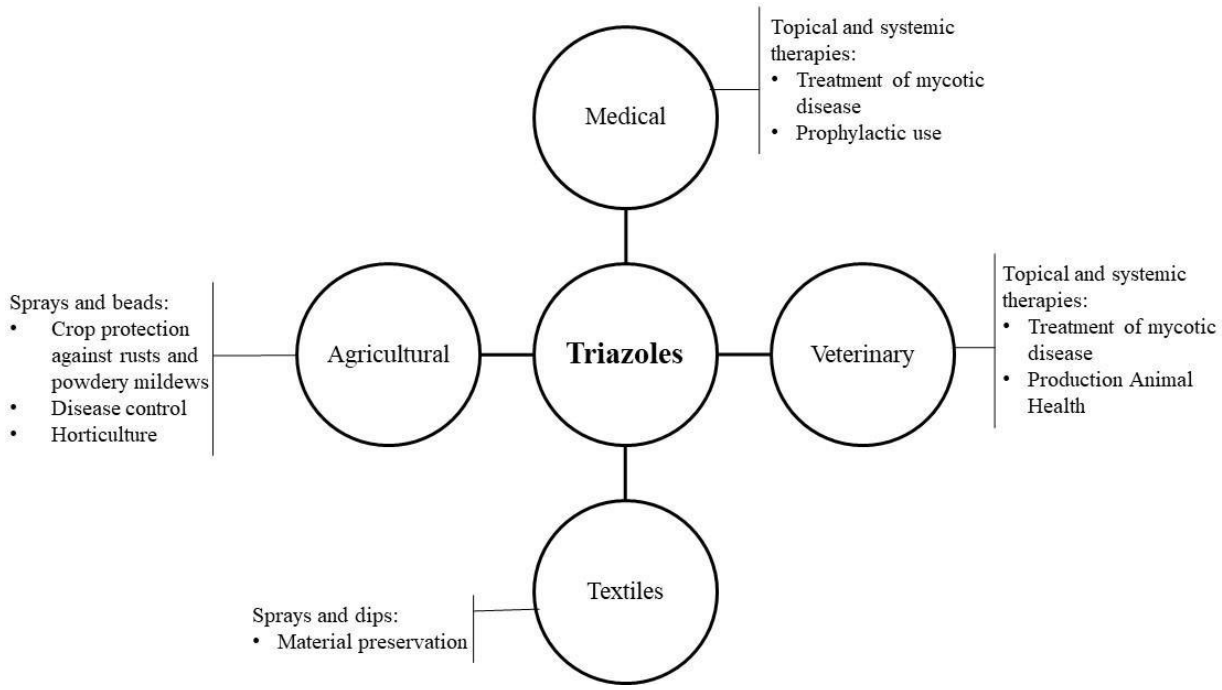
Triazole antifungal drugs are commonly used to control or prevent fungal infections in medical patients (human and veterinary), and fungal contamination of agricultural crops and manufactured materials (Figure 1) (Chowdhary et al., 2017).

Cyp51 is a cytochrome P450 enzyme gene family required for sterol biosynthesis, and is maintained across biological kingdoms, including bacteria, fungi and humans (Hargrove et al., 2015). In fungi it encodes for the demethylation of lanosterol to ergosterol and is important for maintaining the membrane permeability and function of fungal cell walls (Hargrove et al., 2015). There have been two paralogue *Cyp51* genes identified in *A. fumigatus, cyp51A* and c*yp51B* (Mellado et al., 2001). Both genes encode the cytochrome P450 sterol 14α-demethylase, the target protein for antifungal azole drugs that inhibit the formation of sterol precursors and cause the accumulation of 14α-methylated precursors by preventing the binding and oxidation of substrates at the P450 active site (Hargrove et al., 2015). *Cyp51A*

is thought to be induced by the presence of azoles whereas *cyp51B* is thought to be constitutively expressed, and both genes share an amino acid similarity of 59% (Hargrove et al., 2015).

Medical triazoles used to treat aspergillosis in humans include itraconazole, voriconazole, posaconazole and isavuconazole. Agriculturally applied triazole de-methylation inhibitors (DMIs) with similar chemical structure and protein docking mechanisms to these include propiconazole, tebuconazole, epoxiconazole, difenoconazole and bromuconazole (Snelders et al., 2012).

Figure 1. The applications of azole therapeutics to medical, veterinary and agricultural industries.



2.3.2 Antifungal susceptibility testing

In the clinical laboratory, 48-hour incubation microbroth dilution testing is used to determine the susceptibility phenotype of *A. fumigatus* to a number of antifungal drug classes; including minimum inhibitory concentrations (MICs) to azoles and amphotericin B where the MIC is read at 100% inhibition of visual growth; and minimum effective concentrations

(MECs) to echinocandins where the MEC is read as the lowest drug concentration associated with compact (shorter and smaller) hyphal forms compared to the hyphal growth in the positive control well (long and unbranched) (Clinical Laboratory Standards Institute (CLSI), 2017, EUCAST, 2017). These results are then interpreted to determine the susceptibility or resistance of an isolate. Currently two standards are widely accepted for testing, the European Committee for Antibiotic Susceptibility Testing (EUCAST) and Clinical Laboratory Standards Institute (CLSI) M38 (formerly M38-A2) testing methods (EUCAST, 2017, Clinical Laboratory Standards Institute (CLSI), 2017). No interpretive breakpoints have yet been resolved for filamentous fungi for the CLSI method, and this includes *A. fumigatus*. Instead epidemiological cut-off values (ECVs) have been developed based on wild-type *A. fumigatus* isolate susceptibility profiles for detecting azole resistance (Rodriguez-Tudela et al., 2008, Verweij et al., 2009a, Pfaller et al., 2009, Espinell-Ingroff et al., 2010, Espinel-Ingroff et al., 2013, Clinical Laboratory Standards Institute (CLSI), 2017). Proposed breakpoints have been recommended for EUCAST testing based on knowledge of azole therapeutic drug actions, and *A. fumigatus in vivo* and *in vitro* susceptibility results, and these have also been applied to CLSI result interpretation (Table 2) (Verweij et al., 2009a, Wiederhold et al., 2016, EUCAST, 2017).

Commercially available testing kits have also been developed for rapid use, including the strip diffusion tests Etest® (produced by bioMérieux SA, France) and Liofilchem® MIC Strip Test (Liofilchem, Roseto degli Abruzzi, Italy), Sensititre™ YeastOne™ (colorimetric microbroth dilution testing produced by Thermo Scientific Trek Diagnostic Systems, USA) and Vitek® 2 (an automated antifungal susceptibility testing system produced by bioMérieux SA, France). While the Sensititre™ YeastOne™ kits have been demonstrated to yield similar results to the CLSI M38 standard for *Aspergillus fumigatus*, this test and Vitek® 2 have only been approved for testing yeast, not mould fungal species in Europe (Meletiadis et al., 2002, Alastruey-Izquierdo et al., 2015, Mello et al., 2017). The Sensititre™ YeastOne™ test is used by the Australian National Reference Laboratory in Medical Mycology following the preparation standards set for moulds in the CLSI M38 method, as CLSI or EUCAST methods are not routinely available in the region (Ellis, 2017, Kidd et al., 2017, Clinical Laboratory Standards Institute (CLSI), 2017). The reliability of the E-test® to detect resistance has not been validated, with multiple studies reporting varied results on its usefulness in comparison to broth microdilution testing (Meletiadis et al., 2002, Ozkutuk et al., 2008, Lamoth and Alexander, 2015, Arendrup et al., 2017a). A recently accepted manuscript comparing E-test® and the EUCAST microbroth dilution method using *A. fumigatus* clinical isolates from the University

Hospital Münster, Germany, found similar results for both methods for itraconazole, voriconazole and posaconazole (Idelevich et al., 2018). The same study also compared the Liofilchem® MIC Strip Test with EUCAST testing, and while itraconazole and voriconazole results were similar, the MIC Test Strip produced much lower agreement for posaconazole. Liofilchem® MIC Strip Test did however have good agreement with EUCAST for isavuconazole (Idelevich et al., 2018), also supported by another recent study looking at this testing method for *A. fumigatus* isolates (Arendrup et al., 2017a); however, studies on this test are limited. Recently, the development of an azole screening test called the VIPcheck™ (Balis Laboratorium V.O.F., Boven Leeuwen, the Netherlands) test has proven useful and will be discussed later in this chapter.

Table 2. Proposed breakpoints (mg/L) for EUCAST (EUCAST, 2017) and CLSI (Espinell-Ingroff et al., 2010, Clinical and Laboratory Standards Institute (CLSI), 2018) antifungal susceptibility testing.

Testing Method	**Itraconazole (mg/L)**	**Voriconazole (mg/L)**	**Posaconazole (mg/L)**	**Isavuconazole (mg/L)**
EUCAST	≤1 (S) >1 – 2 (I) >2 (R)	≤ 1 (S) >1 – 2 (I) >2 (R)	≤ 0.125 (S) >0.125 – 0.25 (I) >0.25 (R)	≤1 (S) >1 (R)
CLSI	1 (ECV)	1 (ECV)	0.5 (ECV)	1 (ECV)

S= susceptible; I= intermediate; R= resistant; ECV= epidemiological cut-off value

2.3.3 The development and global spread of azole resistance

Acquired defence mechanisms are virulence factors that fungi develop in response to environmental pressures for survival. These acquired or adaptive defence mechanisms enable fungi to survive antifungal treatments and present another challenge in the global battle against antimicrobial resistance. The main acquired defence mechanism of concern amongst *Aspergillus* species is the development of azole resistance. Since first reported in the late 1990s, the number of triazole resistant *A. fumigatus* isolates amongst human patients has

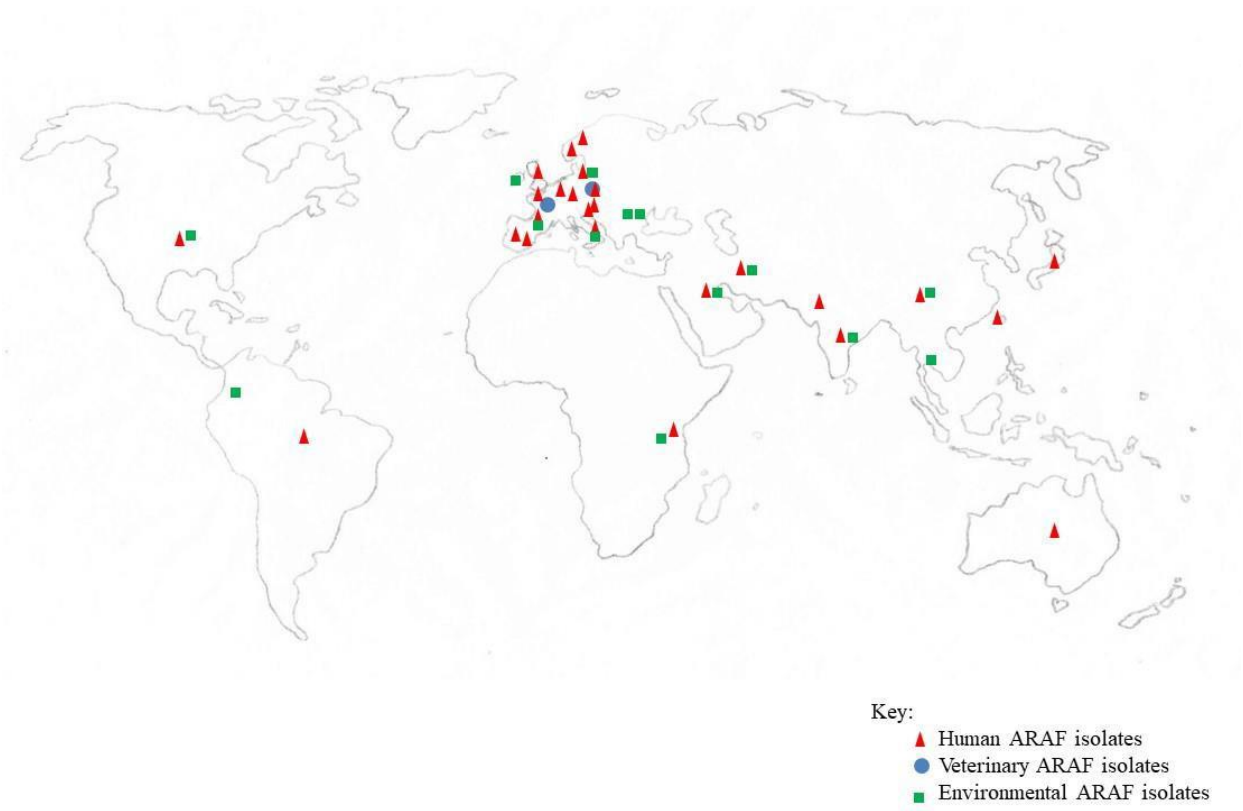
increased globally, particularly in the past decade (Denning et al., 1997, Chryssanthou, 1997, Snelders et al., 2008, Howard et al., 2009, Bueid et al., 2010, Lockhart et al., 2011, van der Linden et al., 2011, Mortensen et al., 2011a, Morio et al., 2012, Burgel et al., 2012, van der Linden et al., 2013, Bader et al., 2013, Seyedmousavi et al., 2013, van der Linden et al., 2015, Lestrade et al., 2016, Mohammadi et al., 2016, Zhao et al., 2016). This presents challenges for patient treatments as there are few approved alternate antifungal drug classes available.

The epidemiology of ARAf varies depending on the country, region and hospital patient group. Hospital and reference laboratory studies on *A. fumigatus* from human clinical patients from around the world have shown an azole-resistance prevalence of 7.9% in Taiwan (Wu et al., 2015), 6.6% in Pakistan (Perveen et al., 2016), 9.1% to 13.8% in Japan (Tashiro et al., 2012a, Tashiro et al., 2012b, Toyotome et al., 2016), 1.8% to 1.9% in India (Chowdhary et al., 2012a, Chowdhary et al., 2015), 3.2 to 4.2% in Iran (Seyedmousavi et al., 2013, Mohammadi et al., 2016), 12.5% in one small study from Kuwait (Ahmad et al., 2015), 4.9% in the United States (Pham et al., 2014), and up to 28% in the United Kingdom (Bueid et al., 2010) and 26% in the Netherlands (van der Linden et al., 2015). The prevalence of ARAf has been most widely investigated in Europe, particularly Western Europe (van der Linden et al., 2015).

A. fumigatus can reproduce sexually, asexually or by parasexual means. Sexual reproduction results in genetic variation via meiotic recombination that may enhance a resistant phenotype, asexual reproduction enables fast propagation of a spontaneous mutation and parasexual recombination can also increase genetic variation via diploidisation allowing mitotic recombination (Verweij et al., 2016, Zhang et al., 2017). Asexual and parasexual processes occur in *A. fumigatus* and most likely contribute to development and spread of azole resistance in human hosts (Verweij et al., 2016), the former being most common and thought to be associated with lung cavity aspergillomas and the latter with IA (Snelders et al., 2008, Verweij et al., 2016, Meis et al., 2016).

The increasing global prevalence of ARAf isolates (Figure 2) threatens successful treatment of aspergillosis patients and is a global public health concern.

Figure 2. World map with regions where azole resistant *Aspergillus fumigatus* has been detected in animals (blue) (Beernaert et al., 2009, Ziolkowska et al., 2014), humans (red) (Verweij et al., 1998, Verweij et al., 2002, Snelders et al., 2008, Howard et al., 2009, Mortensen et al., 2010, Klaassen et al., 2010, Bueid et al., 2010, Alanio et al., 2011, Mortensen et al., 2011a, Mortensen et al., 2011b, van der Linden et al., 2011, Lockhart et al., 2011, Burgel et al., 2012, Morio et al., 2012, Chowdhary et al., 2012a, Tashiro et al., 2012b, Tashiro et al., 2012a, Seyedmousavi et al., 2013, van der Linden et al., 2013, Bader et al., 2013, Pham et al., 2014, Choukri et al., 2015, van der Linden et al., 2015, Wu et al., 2015, Ahmad et al., 2015, Mohammadi et al., 2016, Mushi et al., 2016, Zhao et al., 2016, Alanio et al., 2016, Perveen et al., 2016, Wiederhold et al., 2016, Toyotome et al., 2016), and the environment (green) (Snelders et al., 2009, Mortensen et al., 2010, Chowdhary et al., 2012b, Badali et al., 2013, van der Linden et al., 2013, Chowdhary et al., 2014b, Prigitano et al., 2014, Ahmad et al., 2014, Sharma et al., 2015, Bader et al., 2015, Kano et al., 2015, Le Pape et al., 2016, Jeanvoine et al., 2017, Zhang et al., 2017, Hurst et al., 2017, Tangwattanachuleeporn et al., 2017, Ren et al., 2017, Santoro et al., 2017).



2.3.4 *Cyp51A* mechanisms of azole resistance

In moulds the *cyp51A* gene encodes ergosterol synthesis via the conversion of the cytochrome P450 enzyme lanosterol14-alpha demethylase, an important step in the biosynthetic pathway. Azole drugs target this enzyme, inhibiting the catalytic function of 14-alpha demethylase thus preventing ergosterol formation, leading to the accumulation of sterol precursors (Snelders et al., 2010, Hargrove et al., 2015, Liu et al., 2016). The main azole resistance mechanism is thought to be due to mutations in this gene, achieved by either inhibiting access to the haem protein or by causing instability in the protein complex via gene over-expression or single point mutations, leading to structural changes that inhibit drug binding to the enzyme.

The main mechanisms of *cyp51A* mediated azole resistance include tandem repeats in the promoter region of the gene and/or alterations in the *cyp51A* gene (i.e. point mutations). Environmental acquired mutations include single point mutations most often in combination with a tandem repeat, whereas patient acquired resistance is usually only associated with point mutations (Meis et al., 2016). The TR_{34}/L98H and TR_{46}/Y121F/T289A mutations are thought to be environmentally acquired (Snelders et al., 2009, Verweij et al., 2009b, Mortensen et al., 2010, Vermeulen et al., 2012, van der Linden et al., 2013) and there is evidence of geographical spread of environmentally induced resistance spores in some regions (e.g. from the Netherlands to other European, African and Asian countries) (Snelders et al., 2008, Verweij et al., 2009b, Camps et al., 2012a, Snelders et al., 2012, Vermeulen et al., 2012, van der Linden et al., 2013, Chowdhary et al., 2014b, Hagiwara et al., 2016). The most commonly identified resistance mechanism, TR_{34}/L8H, has been demonstrated to infer pan-azole resistance, while the TR_{46}/Y121F/T289A resistance mechanism leads to voriconazole resistance (Mann et al., 2003, Camps et al., 2011, Rodriguez-Tudela et al., 2008, van der Linden et al., 2013, Chowdhary et al., 2013). Other tandem repeat and tandem repeat / single point mutation combinations have been described. The TR_{53} mechanism was reported in a paediatric patient from the Netherlands with osteomyelitis due to *A. fumigatus*. The patient had chronic granulomatous disease and had been on itraconazole prophylactically. The isolate, sampled in 2006, was itraconazole and voriconazole resistant (Hodiamont et al., 2009). A recently published report from the Netherlands looking at environmental and clinical isolates found new tandem repeats and single point mutations associated with azole resistance, including the TR_{46}^{3}/ Y121F/M172I/T289A/G448S and TR_{46}^{4} /Y121F/M172I/T289A/G448S (Zhang et al., 2017).

Single point mutations are commonly seen in patients with chronic aspergillosis on long-term azole therapy and while 59 single point mutations have been identified, only a small number have been demonstrated to correlate directly with azole resistance (Weber et al., 2017). The single point mutation G54 has been associated with cross resistance to itraconazole and posaconazole (Mann et al., 2003, Camps et al., 2012b, Chowdhary et al., 2014b). The mutation G138 has been associated with itraconazole and voriconazole resistance (Howard et al., 2006, Chowdhary et al., 2014b). Both G54 and G138 have also been associated with pan-azole resistance (Howard et al., 2006, Garcia-Effron et al., 2008, Wiederhold et al., 2016). The mutation G448 is associated with voriconazole resistance and M220 with reduced susceptibility to triazoles with varying patterns reported (Mann et al., 2003, Mellado et al., 2004, Chen et al., 2005, Howard et al., 2006, Howard et al., 2009, Bellete et al., 2010, Peláez et al., 2012). A number of other mutations have been reported amongst both azole-susceptible and azole-resistant *A. fumigatus* isolates, sometimes in association with single point mutations that confer resistance, and sometimes on their own, including F46Y, N248T/K, D255E, E427K and M172V (Howard et al., 2009, Snelders et al., 2010, Escribano et al., 2011, Alanio et al., 2012, Buied et al., 2013, Prigitano et al., 2014, Kidd et al., 2015, Lavergne et al., 2015, Abdolrasouli et al., 2015, Shalhoub et al., 2015, Liu et al., 2015).

A. fumigatus cyp51A protein homology models were developed to help predict the significance of mutations in the gene that might be related to azole resistance (Xiao et al., 2004, Snelders et al., 2010, Alcazar-Fuoli et al., 2011, Fraczek et al., 2011, Hargrove et al., 2015, Liu et al., 2016, Nash and Rhodes, 2017). These have been designed based on bacterial (*Mycobacterium tuberculosis*) (Xiao et al., 2004, Snelders et al., 2010), human (*Homo sapiens*) (Fraczek et al., 2011) and more recently fungal (*Saccharomyces cerevisiae*) (Alcazar- Fuoli et al., 2011, Liu et al., 2016) *cyp51A* protein structures that have similarities with *A. fumigatus cyp51A* protein of 29%, 38% and 50% respectively. Most recently a model of *A. fumigatus cyp51A* has been constructed based on the crystal structure of the *A. fumigatus cyp51B* gene (Hargrove et al., 2015). This demonstrated the highest similarity of 59%. The model has since been used to demonstrate how the TR_{34}/L98H mechanism causes pan-azole resistance (Nash and Rhodes, 2017).

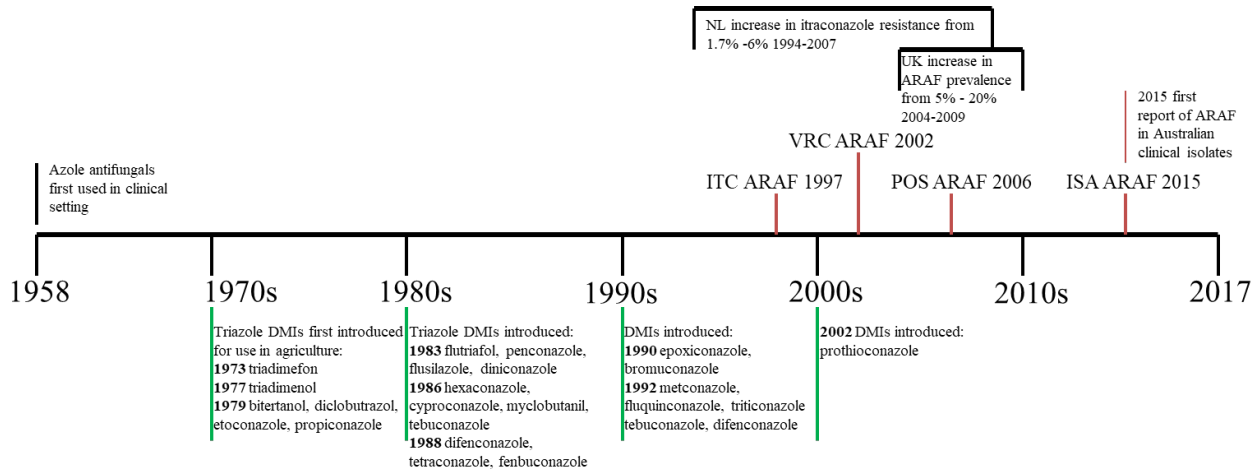
The *cyp51A* gene has been amplified in few *Aspergillus* species, including *A. fumigatus*, *A. flavus* (Mellado et al., 2001) and *A. lentulus* (Mellado et al., 2011). More recently the whole genome sequence of one AVSC species *A. udagawae* was released (Kusuya et al., 2015),

including the *cyp51A* region; however, analysis of this region is not reported. The *cyp51A* gene has not been amplified in any other AVSC species. Thus, whether the reported high antifungal MICs in AVSC species are due to *cyp51A* mediated resistance mechanisms is unknown.

2.3.5 Clinical and environmental azole resistance development mechanisms

The rise and spread of ARAf has been linked to the use of azole class drugs in agriculture and the clinical setting (Figure 3). In medical patients, resistance has been linked to previous exposure to azole class antifungal drugs, either through agricultural practices using azole-based fungicides on crops (i.e. fungicide-driven resistance) (van der Linden et al., 2011), or the repeated use of azole therapeutics clinically (i.e. 'in-patient' resistance development seen amongst CPA, ABPA and CF patients) (Vermeulen et al., 2013, van der Linden et al., 2015). Fungicide-driven resistance appears to be the most commonly acquired mechanism of ARAf, with two-thirds of ARAf patients having no previous exposure to azole therapeutics (van der Linden et al., 2011, Meis et al., 2016). Demethylation inhibitors (DMIs) are azole-class fungicides used commonly in agriculture for several decades to protect crops from fungal disease and ensure food safety (Russell, 2005, Lago et al., 2014, Hollomon, 2017). DMIs are also used in the horticulture, wood and textile industries (Snelders et al., 2009, Hollomon, 2017). In the Australian agricultural industry DMIs are used regularly to control non-*Aspergillus* fungal diseases in plants, including rust and mildew; however, since *A. fumigatus* is ubiquitous, it would be regularly exposed to DMIs (Lago et al., 2014). Azoles can survive in soil for prolonged periods, contributing to resistance development (Mortensen et al., 2010).

Figure 3. Timeline showing the introduction of triazole DMIs for agricultural use (green) (Russell, 2005, Price et al., 2015) and the development of azole resistance in clinical isolates (red) (Snelders et al., 2008, Howard et al., 2009, Bueid et al., 2010, Kidd et al., 2015). Abbreviations: DMI, demethylase inhibitors; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; ISA, isavuconazole; ARAf azole-resistant *Aspergillus fumigatus*; NL, the Netherlands; UK, United Kingdom.



2.3.6 Azole resistance in *A. fumigatus* clinical isolates

Detection of ARAf is important as it can lead to disease progression in patients and poor clinical outcomes (van der Linden et al., 2015), with an increased mortality rate amongst patients with azole-resistant IA (van der Linden et al., 2011, Steinmann et al., 2015). One Dutch study reported a high rate of ARAf in ICU patients with IA, with a 100% mortality rate (Russcher et al., 2014). In the clinical setting antifungal susceptibility testing and/or failure of the patient to respond to therapy alert the clinician to ARAf. More recently rapid DNA testing for azole resistance and *Aspergillus* species confirmation in clinical BAL samples has been developed, detecting the most commonly prevalent mutations (TR_{34}/L98H, T289A and Y121F) and isolates of *A. fumigatus*, *A. terreus* and *Aspergillus* species in clinical isolates (AsperGenius®, PathoNostics, Maastricht, NL) (Chong et al., 2015, Chong et al., 2016, White et al., 2017, Chong et al., 2017, Montesinos et al., 2017, Schauwvlieghe et al., 2017).

ARAf has increased in prevalence throughout the world since the early 2000s, with some regions now reporting a prevalence in human clinical isolates as high as 20-26% (Bueid et al., 2010, van der Linden et al., 2015). Prevalence is highest in Western Europe, but

varies between regions and countries and within hospital populations. Surveillance studies on clinical isolates from Europe, North America, Africa, the Middle East and Asia demonstrate a prevalence of 0 – 26%, depending on the disease syndrome and subsequent azole exposure of the sample population (Verweij et al., 1998, Verweij et al., 2002, Snelders et al., 2008, Howard et al., 2009, Mortensen et al., 2010, Klaassen et al., 2010, Bueid et al., 2010, Alanio et al., 2011, Mortensen et al., 2011a, Mortensen et al., 2011b, van der Linden et al., 2011, Lockhart et al., 2011, Burgel et al., 2012, Morio et al., 2012, Chowdhary et al., 2012a, Tashiro et al., 2012a, Tashiro et al., 2012b, Seyedmousavi et al., 2013, van der Linden et al., 2013, Bader et al., 2013, Pham et al., 2014, Choukri et al., 2015, van der Linden et al., 2015, Ahmad et al., 2015, Mohammadi et al., 2016, Mushi et al., 2016, Zhao et al., 2016, Alanio et al., 2016, Wiederhold et al., 2016, Toyotome et al., 2016, Perveen et al., 2016) (see Table 3 for summary of prevalence studies). Azole resistance has been demonstrated to be widespread in Europe (van der Linden et al., 2015).

2.3.7 Early investigations into azole-resistance in *A. fumigatus* clinical isolates

Itraconazole resistance in isolates of *A. fumigatus* from human clinical patients was first reported in 1997 (Denning et al., 1997). Using the macrobroth dilution technique, Denning et al. (1997) found resistance to itraconazole (MICs >16 mg/L) in three isolates from two patients diagnosed with IA. Both patients were from California, the United States of America, and samples had been collected in 1987 (Patient 1) and 1989 (Patient 2). Both patients had been treated with itraconazole for IA (Denning et al., 1997). Then, only itraconazole and amphotericin B were licensed for therapy in human patients so this news prompted further prevalence studies and the screening for resistance in individual case reports. Shortly after this publication, another study investigating antifungal susceptibility of *Aspergillus* species clinical isolates from hospital patients in Sweden found a prevalence of 3.7% ARAf (itraconazole) in 107 isolates of *A. fumigatus*, with all three patients from whom ARAf were isolated being on itraconazole therapy (Chryssanthou, 1997).

2.3.8 Prevalence of azole-resistant *A. fumigatus* in the Netherlands

Initial prevalence studies in the Netherlands demonstrated that ARAf was uncommon. A study of 130 clinical isolates and 20 environmental *A. fumigatus* isolates from a University Hospital in Nijmegen showed a low prevalence of ARAf of 0.7% (1/150 isolates) with only one

voriconazole resistant clinical isolate (Verweij et al., 1998); however, later phylogenetic studies showed this isolate was the cryptic species *A. lentulus* (Hong et al., 2010). Another ARAf prevalence study in the Netherlands, tested 170 *A. fumigatus* isolates from 1945 to 1998 from 114 patients at 21 different hospitals (Verweij et al., 2002) for susceptibility to itraconazole and voriconazole using the NCCLS M38-P and E-test methods. It showed a low prevalence of azole resistance during this period (3/170 isolates, 1.8%). All three resistant isolates were from the same lung-transplant recipient on itraconazole therapy for respiratory tract aspergillosis. All other isolates tested were susceptible to itraconazole, and all isolates were susceptible to voriconazole (Verweij et al., 2002).

The first ARAf report in 1997 (Denning et al., 1997) and the initial prevalence studies of clinical *A. fumigatus* isolates in the Netherlands indicated that ARAf was uncommon (Verweij et al., 1998, Verweij et al., 2002). However, reports of clinical cases increased throughout the first decade of the 2000's and additional prevalence studies of retrospective cases demonstrated an increase in ARAf. This prompted further investigations from different countries to determine the extent of the problem. Initially the TR_{34}/L98H mutation was identified as the cause of ARAf; however, with more cases and studies, more *cyp51A* resistance mechanisms conferring resistance were discovered, although TR_{34}/L98H remains the most common mutation (Verweij et al., 2007).

The highest number of prevalence studies of ARAf among human clinical isolates have been performed in the Netherlands, with prevalence rates ranging from 1.8% to 26% (Verweij et al., 2002, Snelders et al., 2008, Klaassen et al., 2010, van der Linden et al., 2011, van der Linden et al., 2013, van der Linden et al., 2015). Between 64% and 71% of patients in the Netherlands with ARAf due to environmental resistance mechanisms had no prior treatment with azole drugs (van der Linden et al., 2011, van der Linden et al., 2013). A prevalence of 5.3% itraconazole resistance was found in clinical *A. fumigatus* isolates collected from seven University Medical Centres from 2007 to 2009 (van der Linden et al., 2011). The most common mutation was TR_{34}/L98H and most ARAf isolates were from azole-naïve patients (64%). Other mutations included G54W (itraconazole and posaconazole resistant), P216L (itraconazole resistant), F219I (itraconazole resistant), and F46Y, G89G, M172V, N248T, D255E, L358L, E427K, C454C (itraconazole and voriconazole resistant). Resistant isolates

were most common amongst patients with haematologic or oncologic conditions (van der Linden et al., 2011).

Another study investigating clinical isolates from hospital patients received at the Radboud University Nijmegen Medical Center in the Netherlands, collected from 1994 to 2007, found a prevalence of ARAf of 2.6% (32/1219 hospital patients), with the first resistant isolate detected in 1998, and the remainder post 2000, increasing in prevalence yearly from 1.7% to 6% (Snelders et al., 2008). Most of these resistant isolates had the *cyp51A* TR_{34}/L98H mutation (n=30); one had the single point mutation M220V and one had no *cyp51A* mutations. The patient population varied with the highest incidence of itraconazole resistance reported from haematology (25%), oncology (15.6%), and lung disease (cystic fibrosis, chronic obstructive pulmonary disease) (15.6%) patients (Snelders et al., 2008). This study also included isolates from 28 other medical centres in the Netherlands from 1994 to 2006, finding an ARAf prevalence of 12.8% (13/101 patients), and patient isolates from Belgium, France, Sweden, Norway, United Kingdom (isolate had originated in the US) and Greece from 1994 to 2002, with a prevalence of 1.9% (6/317 isolates). *Cyp51A* mutations were found in one isolate each from France (M220R), Sweden (M220I) and Norway (TR_{34}/L98H) and in 10 isolates from the Netherlands (M220I (n=1), TR_{34}/L98H-S297T-F495I (n=1), TR_{34}/L98H-S297T (n=1) and TR_{34}/L98H (n=7))
(Snelders et al., 2008).

A lower prevalence of 2% ARAf (itraconazole) was found in other studies from the Netherlands (Klaassen et al., 2010; Verweij et al., 2002). Verweij et al. (2002) looked at isolates collected from 1945 to 1998 and only found resistance in isolates collected after 1990. A more widespread rate of ARAf of 6.8% (63/921 patients) was found in clinical isolates of *A. fumigatus* collected from 2009 to 2011 from eight university hospitals in the Netherlands (van der Linden et al., 2013). Amongst the resistant isolates, 74.6% (47/63) had multi-azole resistance and the TR_{34}-L98H mechanism, 20.6% (13/63) had voriconazole resistance and the TR_{46}/Y121F/T289A, with one isolate additionally having the M172I and G448S substitutions, and 4.7% (3/63) had no *cyp51A* mutations. Interestingly, isolates with the TR_{46}/Y121F/T289A mechanism were also found to be tebuconazole resistant (van der Linden et al., 2013).

A national survey undertaken in 2015 investigating azole resistance rates in clinical isolates from eight University Medical Centres and eight non-academic teaching hospitals in the Netherlands reported a prevalence of ARAf of between 2% and 30%, with an average resistance rate of 5% (Lestrade et al., 2016). High risk wards included haematology units, where azole drugs itraconazole, voriconazole and posaconazole were used for prophylaxis. Of the centres, 81.3% used voriconazole as the primary first-line therapy for treating patients with IA, with 87.5% using liposomal amphotericin B when ARAf was diagnosed. Another recent study investigating the prevalence of ARAf in patients with haematological malignancies presenting to the Radboud University Medical Center from 2006 – 2012 found a lower resistance rate (overall 1.2%, 5/432 patients), including a low voriconazole resistance rate of 0.5% (Lestrade et al., 2018).

2.3.9 Prevalence of azole-resistant *A. fumigatus* in the United Kingdom

A United Kingdom study of 519 isolates collected from patients with aspergillosis from 1992 to 2007 found an overall prevalence of ARAf of 7% (Howard et al., 2009). While the first ARAf isolate was from 1999, a significant increase in prevalence was observed in isolates from 2004 (5% ARAf) to 2007 (17% ARAf). Two isolates had the TR_{34}/L98H mechanism and were pan-azole resistant, while several non-synonymous single point mutations were associated with resistance. This included mutations conferring itraconazole only resistance (P216L), itraconazole and voriconazole resistance (F46Y/M172V/E427K, F46Y/M172V/E427K/N248T/D255E, H147Y, Y431C, G434C, G448S/H147Y (and in the case of this last mutation combination, one case also of posaconazole resistance)), itraconazole and posaconazole resistance (G54E/R/V), voriconazole and posaconazole resistance (M220K), voriconazole and variable posaconazole resistance (M220T) and pan-azole resistance (G138C) (Howard et al., 2009). This high prevalence continued, and in 2010 a combined prevalence of 28% (64/230 isolates) was reported amongst human derived clinical *A. fumigatus* isolates submitted to the Mycology Reference Centre Manchester, United Kingdom from 2008-2009 (Bueid et al., 2010). The incidence of ARAf increased from 14% in isolates from 2008 to 20% in isolates from 2009. Susceptibility testing was performed using the EUCAST method. Most of these ARAf isolates were multi-azole resistant (50/64, 78%), with voriconazole resistance less common (2/64, 3%). Itraconazole resistance was most common, detected in 97% (62/64) of isolates. *Cyp51A* sequencing revealed mutations in 67% of ARAf isolates, including M220I/K/V/R/W, A284T, the F46Y/G89G/M172V/L358L/E427K/C454C

combination, and the F46Y/G89G/M172V/L358L/E427K/C454C/N248T/D255E combination. The remaining 43% had wild-type *cyp51A*, suggesting other mechanisms were responsible for resistance in this group (Bueid et al., 2010). Another study from the United Kingdom investigating azole susceptibility (itraconazole, voriconazole and posaconazole) of colonies of *A. fumigatus* from aspergillomas of three patients with CPA found azole-resistant isolates in one patient, and a mixture of azole-susceptible and azole-resistant isolates in another. The patient with only azole-resistant isolates had *cyp51A* mutations, including M220K and M220T (Howard et al., 2013).

2.3.10 Multi-centre international surveillance studies

The ARTEMIS surveillance program was established to detect global ARAf trends, with 100 participating medical centres around the world (Lockhart et al., 2011). It included a large ARAf prevalence study on 497 isolates of *A. fumigatus* from 2008 to 2009 from 62 medical centres worldwide. Testing susceptibility to itraconazole, voriconazole and posaconazole using the CLSI M38-A2 method showed a prevalence of ARAf of 5.8% (29/497) with resistant isolates from China (n=24), the Czech Republic (n=2), Portugal (n=1), the United States (n=1) and Brazil (n=1). Eight isolates from China with itraconazole resistance harboured the TR_{34}/L98H mutation plus the mutations S297T and F495I, and interestingly these isolates did not display cross- resistance to voriconazole, as is typical of isolates harbouring the TR_{34}/L98H mutation. Additionally, not all resistant isolates had *cyp51A* mutations, and the mutations F47Y/M172V/N248T/D255E/E427K were seen amongst susceptible wild-type isolates as well as isolates with elevations in MICs for posaconazole. This study was the first to document ARAf due to TR_{34}/L98H outside of Europe, in clinical isolates from China (Lockhart et al., 2011). This study also included antifungal susceptibility data for three Australian derived isolates, all with wild-type ECVs and *cyp51A* sequences (Lockhart et al., 2011).

A recent international study involving medical centres from 19 countries (18 European and 4 non-European sites) found a prevalence of ARAf in isolates of *A. fumigatus* of 3.2 %, with 47 *A. fumigatus* isolates demonstrating itraconazole resistance (van der Linden et al., 2015). The TR_{34}/L98H was the most common resistance mechanism, and was found amongst isolates from Austria, Belgium, Denmark, France, Italy and the Netherlands. Other mechanisms reported

included the TR_{46}/Y121F/T289A from the Netherlands, F46Y/M172G with TR_{34}/L98H from Belgium, G54W with TR_{34}/L98H from France, F46Y/M172G from Sweden and from the United Kingdom the mutations P381R/D481E, L77V/L399I/D481E, M220I, M220R, G54R, G54E and G54W were found (van der Linden et al., 2015).

2.3.11 Prevalence of azole- resistant *A. fumigatus* in patients with cystic fibrosis and haematologic disorders in France, Denmark and Germany

An ARAf prevalence rate of 0.9% - 8% has been reported amongst clinical isolates from France, higher amongst patients with CF, and lower amongst patients with haematological disorders. This may be because azole treatments in haematology patients are generally shorter than the lengthy treatments for chronic lung conditions. One study investigating 118 isolates of *A. fumigatus* collected from 89 patients with haematological malignancies and probable or proven IA from 2006 to 2009 found an ARAf prevalence of 0.9% (Alanio et al., 2011). One isolate had high itraconazole MICs and the G432S mutation. The patient was naïve to azole treatment. The same group reported results at the same institution for *A. fumigatus* isolates recovered from 2012 - 2014 from French patients with probable or proven IA, demonstrating a similar low prevalence of ARAf of 1.5% (1/68 isolates) (Alanio et al., 2016). The resistant isolate harboured the TR_{34}/L98H resistance mechanism (de Fontbrune et al., 2014). Most isolates came from patients with haematologic malignancies. Another study found a resistance rate of 1.8% (3/165 isolates) amongst patients with chronic respiratory disease (immunocompetent and immunocompromised patients) attending two tertiary hospitals in 2012, with all three resistant isolates demonstrating pan-azole resistance and the TR_{34} /L98H resistance mechanism (Choukri et al., 2015). Recent itraconazole exposure was also associated with high ARAf in another study investigating patients with CF (Burgel et al., 2012). The study investigated ARAf amongst French CF patients in isolates collected from 2010 to 2011, finding a prevalence of 4.6% (6/131 isolates). All six isolates had reduced susceptibility to itraconazole and posaconazole, and 3/6 had reduced susceptibility to voriconazole. Five of the isolates were from patients that had received itraconazole therapy within the preceding three years, and four of these had *cyp51A* mutations reported including G54E, M220I, M220R and TR_{34}/L98H. The remaining resistant isolate was from an azole-naïve patient and harboured the TR_{34}/L98H resistance mechanism. It was proposed that the high prevalence of ARAf in CF patients was due to the use of prolonged courses of itraconazole for treating ABPA amongst this cohort

(Burgel et al., 2012). Another study screening for ARAf in respiratory samples from patients at risk of respiratory aspergillosis found ARAf in 1/19 isolates (5.3%) (Zhao et al., 2016). The isolate had pan-azole resistance and the TR_{34}/L98H resistance mechanism was found on *cyp51A* analysis. The patient had been on posaconazole therapy for one year (Zhao et al., 2016). An even higher prevalence of itraconazole resistance of 8% was reported in another study of French CF patients (4/50 patients), with isolates collected from 2010 to 2011 (Morio et al., 2012). *Cyp51A* resistance mechanisms found included TR_{34}/L98H (n=5, multi-azole resist), M220T (n=4, itraconazole and posaconazole resistance) and G54R (n=1, itraconazole and posaconazole resistance). Three isolates from one patient were shown to be genetically distinct (Morio et al., 2012).

A study from Denmark examining ARAf in CF patients found a prevalence of resistance to itraconazole of 4.5% (6/133 isolates) (Mortensen et al., 2011a). Five of the resistant isolates had *cyp51A* mutations (M220K; TR34/L98H; TR34/L98H-S297T-F495I, M220I-V101F and Y431C).

A German study investigating ARAf found a prevalence of 3.2% (17/527 isolates) amongst clinical patients, with the highest prevalence detected amongst CF patients (5.2%) (Bader et al., 2013). Reported *cyp51A* resistance mechanisms included TR_{34}/L98H (itraconazole resistant), G54W (itraconazole and posaconazole resistant), M220I (itraconazole resistant) and F219C (itraconazole resistant).

2.3.12 Prevalence studies of azole-resistant *A. fumigatus* external to Western Europe

The USA

Although the first reported isolates of ARAf were sampled from the USA in the 1980s, further investigations into the prevalence of ARAf in the USA were more recent. In 2014 a study investigated azole resistance amongst 1026 *A. fumigatus* isolates collected from 22 states between 2011-2013, and of 51 isolates (51/1026, 4.9%) with MICs above the ECV, only one isolate with an azole resistance associated *cyp51A* mutation M220I was identified (Pham et al., 2014). The isolate had an elevated MIC for itraconazole (reported as $\geq$32 mg/L). Seventeen other isolates with elevated MICs had other mutations not recognised to be associated with resistance. Interestingly the most commonly reported azole resistance

associated *cyp51A* mutation, TR_{34}/L98H was not recovered (Pham et al., 2014). Another US study performed in 2016 targeted clinical isolates with high azole MICs and found numerous *cyp51A* single point mutations as well as tandem repeats in the promoter region of the gene (Wiederhold et al., 2016). Mechanisms associated with itraconazole resistance included TR_{34}/L98H, M220V/I/K, G54R/E, G138S/C, G448S and F219S; with voriconazole resistance G448S (V); with cross resistance to itraconazole and voriconazole G448S; and with cross resistance to itraconazole and posaconazole TR_{46}/Y121F/T289A and G54W/R (Wiederhold et al., 2016).

India

ARAf was first reported in India in 2012 in human patients suspected of having bronchopulmonary aspergillosis, following a screening of 103 *A. fumigatus* isolates from 85 patients collected from 2005 to 2010 (Chowdhary et al., 2012a). Susceptibility to itraconazole, posaconazole, voriconazole and isavuconazole was tested. An ARAf prevalence of 1.9% was reported (2/103 isolates), with multiple triazole resistance and TR_{34}/L98H mutations confirmed in both isolates. Microsatellite typing demonstrated divergence from Dutch ARAf isolates with the same mutation. Both patients were naïve to azole therapeutics (Chowdhary et al., 2012a). Another study investigating *A. fumigatus* isolates from one Indian hospital during 2011 to 2014 found a similar prevalence of ARAf of 1.8% (12/685 isolates) (Chowdhary et al., 2015). Eleven of the ARAf isolates were pan-azole resistant, and one demonstrated resistance to itraconazole and posaconazole. Of the pan-azole resistant isolates, 10 harboured the TR_{34}/L98H mutation, while one had the mutations F46Y/D255E/M172V. The remaining multi- azole resistant isolate harboured the G54E mutation. Microsatellite genotyping of the TR_{34}/L98H isolates demonstrated a unique genotype compared to TR_{34}/L98H isolates from China, the Middle East and Europe. Additionally, all 12 resistant isolates were also resistant to the azole fungicides bromuconazole, cyproconazole, difenoconazole, epoxiconazole, penconazole, tebuconazole, triadimefon, hexaconazole and tricyclazole (Chowdhary et al., 2015).

Iran

ARAf has been reported in Iran. An investigation of 124 clinically relevant isolates of *A. fumigatus* collected from human patients from 2003-2009 revealed a prevalence of ARAf of

3.2% (4/124), with the first azole resistant isolate from 2005 (Seyedmousavi et al., 2013). The TR_{34}/L98H mutation was found in three of four resistant isolates, with no *cyp51A* resistance mechanisms detected in one isolate. ARAf isolates were from patients with pulmonary aspergillosis (n=3) and allergic bronchopulmonary aspergillosis (n=1). Microsatellite genotyping of the isolates showed they were genetically distinct from European strains (Seyedmousavi et al., 2013). A second study from Iran investigated the prevalence of ARAf in clinical isolates from Iran and found a similar prevalence of 3.5% (6/172 *A. fumigatus* isolates) (Mohammadi et al., 2016). The six isolates were resistant to itraconazole and voriconazole and harboured the TR_{34}/L98H mechanism. Microsatellite genotyping revealed these isolates had distinct genotypes to other Iranian and Dutch isolates harbouring the TR_{34}/L98H mechanism (Mohammadi et al., 2016).

Taiwan

A study of *A. fumigatus* isolates collected from two hospitals in Taiwan from 2011 to 2014 demonstrated a prevalence of ARAf of 6.5% (2/31 patients) (Wu et al., 2015). The three ARAf isolates were recovered from respiratory samples from two patients with pulmonary aspergillosis, and both were azole-naïve. Both isolates demonstrated multi-azole resistance to itraconazole, voriconazole and posaconazole and were also cross resistant to tebuconazole and penconazole, two azole fungicides used in agriculture. *Cyp51A* sequencing demonstrated all three isolates harboured the TR_{34}/L98H mutation, and microsatellite genotyping showed one isolate was closely related to ARAf isolates harbouring the same mutation from the Netherlands, India, Iran and Australia, while the other two isolates clustered with other wild-type isolates included in the study, being genetically distinct from ARAf isolates found elsewhere in the world (Wu et al., 2015).

Japan

A study of isolates collected from 1994 to 2010 in one hospital in Japan reported a prevalence of ARAf of 13.8% (27/196 *A. fumigatus* isolates), and *cyp51A* G54E/W/R mutations were found in 14 isolates with resistance to itraconazole (n=9) and posaconazole (n=5) (Tashiro et al., 2012b). A selection of these isolates demonstrated acquired resistance due to the G54E mutation in one isolate from a CPA patient where, compared to earlier azole susceptible isolates, following itraconazole therapy, itraconazole and

posaconazole resistance and the mutation had developed (Tashiro et al., 2012a). Another study from Japan examining clinical and environmental isolates of *A. fumigatus* for ARAf found a prevalence of 9.1% (2/22 isolates) at one hospital amongst isolates collected from 2013 to 2015. Resistance to voriconazole was detected in all ARAf isolates, and one was found to harbour the G448S mutation. This isolate was sampled from a patient that had been treated with voriconazole for 85 days. Interestingly an earlier isolate collected prior to voriconazole treatment in this patient was azole susceptible (Toyotome et al., 2016).

Tanzania

A Tanzanian study investigating azole resistance amongst clinical isolates of fungi causing suppurative otitis media found five isolates of *A. fumigatus* all resistant to itraconazole with reduced susceptibility to posaconazole and voriconazole and harbouring the TR_{34}/L98H mutation (Mushi et al., 2016).

Poland

In Poland Nawrot et al., 2018 found ARAf in 5/121 *A. fumigatus* clinical isolates (4.1%), with all isolates harbouring the TR34/L98H mechanism and demonstrating itraconazole resistance. Four of these isolates had cross-resistance to posaconazole and one isolate had cross-resistance to voriconazole (Nawrot et al., 2018).

Australia

There are limited azole resistance surveillance studies on Australian *A. fumigatus* isolates, with a resistance rate of 0% (van der Linden et al., 2015) to 2.2% (Kidd et al., 2015). In a study by Kidd and others (2015), *cyp51A* sequencing of resistant *A. fumigatus* isolates found the environmentally acquired resistance mechanism TR_{34}/ L98H in two isolates, and single point mutations in four isolates (G54R, F46Y, Y431S, G448S respectively). Other single point mutations associated with the periphery of the protein were considered likely insignificant in terms of azole resistance (M172V, N248T, D255E, and E427K). Three resistant isolates had no *cyp51A* mutations. This study noted that one of the isolates harbouring the TR_{34}/L98H mutation was potentially contracted in Europe; however, microsatellite typing

showed no relatedness to European or the other Australian isolates (Kidd et al., 2015). The other isolate could have been acquired from suburban Sydney. The authors called for further Australia-wide surveillance studies (Kidd et al., 2015).

Table 3. Summary of isolate details from clinical prevalence studies investigating azole resistant *Aspergillus fumigatus*.

Study	**Country**	**No. hospitals**	**No. isolates tested**	**No. isolates yielding Af (%)**	**No. isolates yielding ARAf/Total Af isolates tested (%)**	**No. patients with ARAf/Total patients with Af**
Chryssanthou 1997	Sweden	NS	122	107/122 (87.7%)	4/107 (3.7%)	NS
Verweij et al., 2002	The Netherlands	21	170	170/170 (100%)	3/170 (1.8%)	1/114 (0.9%)
Snelders et al., 2008	The Netherlands	1	1219	1219/1219 (100%)	41/1219 (3.4%)	32/1219 (2.6%)
Snelders et al., 2008	The Netherlands	28	147	147/147 (100%)	22/147 (14.9%)	13/101 (12.8%)
Snelders et al., 2008	Belgium, France, Sweden, Norway, United Kingdom#, Greece	NS	317	317/317 (100%)	6/317 (1.9%)	NS
Howard et al., 2009	United Kingdom	NS	519	519/519 (100%)	34/519 (6.6%)	NS
Klaassen et al., 2010	The Netherlands	14	209	209/209 (100%)	4/209 (1.9%)	4/209 (1.9%)
Buied et al., 2010	United Kingdom	NS	230	230/230 (100%)	64/230 (27.8%)	NS
Alanio et al., 2011	France	1	118	118/118 (100%)	1/118 (0.85%)	1/89 (1.12%)
Lockhart et al., 2011	Multi-centre international (ARTEMIS)	62	497	497/497 (100%)	29/497 (5.8%)	NS
Mortensen et al., 2011a	Denmark	1	133	NS	NS	6/133 (4.5%)
Mortensen et al., 2011b	Denmark	4	NS	NS	NS	2/112 (1.8%)
van der Linden et al., 2011b	The Netherlands	7	2062	1792/2062 (86.9%)	82/1792 (4.6%)	63/1192 (5.3%)

Morio et al., 2012	France	1	85	85/85 (100%)	9/85 (10.6%)	4/50 (8%)
Burgel et al., 2012	France	1	131	285/285 (100%)	6/285 (2.1%)	6/131 (4.6%)
Chowdhary et al., 2012a	India	1	103	103/103 (100%)	2/103 (1.9%)	2/85 (2.35%)
Tashiro et al., 2012b	Japan	1	196	196/196 (100%)	27/196 (13.8%)	NS
Bader et al., 2013	Germany	NS	527	527/527 (100%)	17/527 (3.2%)	NS
Seyedmousavi et al., 2013	Iran	NS	124	124/124 (100%)	4/124 (3.2%)	NS
van der Linden et al., 2013	The Netherlands	7	1315	1315/1315 (100%)	NS	63/921 (6.8%)
Pham et al., 2014	USA	NS	1026	1026/1026 (100%)	51/1026 (4.9%)	NS
Ahmed et al., 2015	Kuwait	NS	16	16/16 (100%)	2/16 (12.5%)	NS
Choukri et al., 2015	France	2	165	165/165 (100%)	3/165 (1.8%)	3/134 (2.2%)
Chowdhary et al., 2015	India	2117	1	685/2117 (32.4%)	12/685 (1.8%)	NS
Kidd et al., 2015	Australia	NS	418	414/418 (99%)	9/414 (2.17%)	NS
van der Linden et al., 2015	Multi-centre international (SCARE-network)	22	3788	NS	NS	46/1450 (3.2%)
Wu et al., 2015	Taiwan	2	38	38/38 (100%)	3/38 (7.9%)	2/31 (6.5%)
Mohammadi et al., 2016	Iran	NS	172	172//172 (100%)	6/172 (3.5%)	6/142 (4.23%)
Perveen et al., 2016*	Pakistan	NS	210	210/210 (100%)	14/210 (6.6%)	NS
Mushi et al., 2016	Tanzania	1	54	5/54 (9.3%)	5/5 (100%)	5/44 (11.4%)
Alanio et al., 2016	France	1	68	68/68 (100%)	1/68 (1.5%)	NS
Zhao et al., 2016	France	1	20	19/20 (95%)	1/19 (5.3%)	NS
Toyotome et al., 2016	Japan	1	22	22/22 (100%)	2/22 (9.1%)	NS

Lestrade et al., 2018	The Netherlands	1	25	25/25 (100%)	5/25 (20%)	5/432 (1.2%)
Nawrot et al., 2018	Poland	NS	121	121/121 (100%)	5/121 (4.1%)	5/109 (4.6%)

Abbreviations: Af, *Aspergillus fumigatus*; ARAf, azole resistant *Aspergillus fumigatus*; NS, not stated. *abstract only

2.3.13 Individual case reports of azole-resistant *A. fumigatus*

Individual cases of ARAf have been reported throughout the world.

Phenotypic and genotypic changes in serial isolates of *A. fumigatus* from a pulmonary tuberculosis patient with pulmonary aspergillosis in China receiving itraconazole therapy (Chen et al., 2005) demonstrated a change in *in vitro* MICs for itraconazole within six months of commencing treatment, from 0.25 mg/L prior to commencing therapy, to >16 mg/L. Two months after treatment stopped, the MIC had reduced again to 0.5 mg/L. Therapy was re-started and three more isolates were obtained over four to seven months, with a return to elevated MICs of >16 mg/L (Chen et al., 2005). *Cyp51A* gene sequencing was performed on all four resistant isolates, and the single point mutation M220I was identified in the first resistant isolate, with G54R identified in the remaining three resistant isolates. Internal transcribed spacer (ITS) region and random amplified polymorphic DNA (RAPD) assay genotyping demonstrated all isolates were identical, indicating they were likely the same *A. fumigatus* strain responding to different conditions induced through the disease course and treatment (Chen et al., 2005). However, other studies have since demonstrated co-infection with azole susceptible and azole resistant strains of *A. fumigatus* (de Valk et al., 2007, Kolwijck et al., 2016).

Multi-azole resistance was reported in a United Kingdom patient treated with itraconazole for over three years then voriconazole for over a year for cavitary aspergillosis with aspergillomas (Howard et al., 2006). Six isolates of
A. fumigatus, collected over 17 months following prolonged treatment with both drugs, consistently demonstrated high MICs of itraconazole and voriconazole. *Cyp51A* sequencing on the first two isolates revealed the G138C mutation. The patient then received caspofungin for over 12 months until dying of respiratory failure attributed to infection (Howard et al., 2006).

A Belgian patient with multiple myeloma (Vermeulen et al., 2012) and mould-active azole-naïve was diagnosed with pulmonary IA. CLSI M38-A2 protocol susceptibility testing showed the sampled isolate was voriconazole resistant (>16 mg/L) and had elevated MICs for itraconazole (4 mg/L) and

posaconazole (1 mg/L) (Vermeulen et al., 2012). The patient was initially treated with an azole antifungal as part of a double-blinded clinical trial but was withdrawn from the trial following poor clinical response and started on liposomal amphotericin B. IA then spread to involve the eye and brain and the patient died. The isolate harboured the TR_{46}/Y121F/T289A mutation. This mutation, first discovered in 2011 in clinical *A. fumigatus* isolates from the Netherlands (Camps et al., 2011), is associated with higher MICs of voriconazole compared to itraconazole, which contrasts with the TR_{34}/L98H mutation that generally has higher MICs for itraconazole (Vermeulen et al., 2012).

The TR_{46}/Y121F/T298H *cyp51A* resistance mechanism was also identified in two case reports from French patients (Lavergne et al., 2015, Lavergne et al., 2017). One reported itraconazole and voriconazole resistance in a CF patient with a history of itraconazole and voriconazole exposure and found the same mutation in soil isolates from a wheat field in the region but not close to the patient's home (Lavergne et al., 2015). The other report, which described a patient with IA infected by an *A. fumigatus* isolate with high MICs of itraconazole and voriconazole, found genotypically identical isolates at the patient's home (garden and house) four months after the patient had died (Lavergne et al., 2017). The patient lived in an agricultural region, and nearby crops found ARAf harbouring the TR_{34}/L98H mutation, with a high prevalence of ARAf noted of 19% (38/200 *A. fumigatus* isolates) (Lavergne et al., 2017). The TR_{46}/Y121F/T289A resistance mechanism has also been reported associated with voriconazole resistance in patients from Denmark (Astvad et al., 2014), Germany (Fischer et al., 2014), Spain (Peláez et al., 2015) and Japan (Hagiwara et al., 2016).

ARAf was first described in a German patient in 2012 (Rath et al., 2012). The patient had been diagnosed with aspergilloma and was treated with oral voriconazole for nine months prior to presentation for lung lobectomy due to treatment failure. ARAf was recovered from a sputum sample, with resistance to itraconazole ($\geq$16mg/L) and voriconazole (4mg/L), and intermediate susceptibility to voriconazole (0.5 mg/L), with the mutation TR_{34}/L98H identified on *cyp51A* sequencing (Rath et al., 2012). A second report from the same year described an acute myeloid leukaemia patient with IA also due to ARAf. The patient had been on a short course of posaconazole treatment (14 days) when the pan-azole resistant sample was isolated. The isolate also harboured the TR_{34}/L98H mutation (Hamprecht et al., 2012).

The first Spanish case of ARAf due to the TR_{34}/L98H mechanism was also isolated from a patient with IA on long-term azole therapy (Mellado et al., 2013). This patient also had chronic myeloid leukaemia and had received an allogeneic hematopoietic cell transplantation. Multiple isolates of *A. fumigatus* were obtained from the patient during treatment. Initially the first isolate was susceptible to azole therapeutics, however *in vitro* testing on a second isolate collected after nine months of azole therapy (one month itraconazole and eight months voriconazole therapy) demonstrated resistance, and a third azole resistant *A. nidulans* isolate was collected after twelve months of voriconazole therapy (Mellado et al., 2013). It is unknown if resistant isolates were due to chronic therapy or if different strains of azole-susceptible and azole-resistant *A. fumigatus* and *A. nidulans* were already present in the patient (i.e. acquired environmentally) and azole treatment selected for the latter. The environmentally associated *cyp51A* mutation TR_{34}/L98H was, however, found in the resistant *A. fumigatus* isolate. The authors suggested that any patient on long-term azole therapy should have regular isolate sampling and antifungal susceptibility testing performed to monitor for the development of azole resistance (Mellado et al., 2013).

A patient from Japan on long-term itraconazole therapy for pulmonary aspergillosis with itraconazole resistance harboured a G54 *cyp51A* mutation (Tashiro and Izumikawa, 2016), and the TR_{34}/L98H mutation was also found in an azole-naïve cancer patient from Japan being treated for pulmonary aspergillosis (Toyotome et al., 2017). The TR_{34}/L98H mechanism has also been found in azole-naïve and azole-exposed IA patients in Denmark (Astvad et al., 2014).

Most recently ARAf has also been reported in South America (Argentina) in a human patient with no previous azole exposure suffering fungal keratitis. The isolate was itraconazole resistant and harboured the G54E mutation (Leonardelli et al., 2017).

2.3.14 Antifungal susceptibility profiles in infections involving multiple *A. fumigatus* genotypes

Multiple genotypes of *A. fumigatus* have been isolated from infected lung lesions, further complicating successful disease control (de Valk et al., 2007). Different azole profiles (resistant and susceptible) have been identified in isolates of *A. fumigatus* collected at the same time from the same patient. This may be due to environmentally acquired infection or from azole-therapy induced selection.

Voriconazole resistant and susceptible *A. fumigatus* coinfection was reported amongst patients in the Netherlands (Kolwijck et al., 2016). One patient with signs of respiratory and renal insufficiency, that was started on voriconazole therapy but died of multiorgan failure within 24 hours of presentation to an ICU unit, had two pan-azole resistant isolates harbouring the TR_{34}/L98H mutation (from BAL and left lung tissue), and one isolate that was azole susceptible (tissue right lung) with wild-type *cyp51A*. Another patient with IA that presented with pneumonia following renal transplantation for diabetic nephropathy was treated with voriconazole after culturing a voriconazole susceptible *A. fumigatus* isolate from a BAL sample (Kolwijck et al., 2016). Ten days after commencing therapy, two bronchial aspirate samples were cultured, one being susceptible to voriconazole with wild-type *cyp51A*, the other being a voriconazole-resistant *A. fumigatus* isolate sampled harbouring the TR_{46}/Y121F/T289A mutation. The patient died after just over a month of therapy with voriconazole and anidulafungin, and at autopsy wild-type *cyp51A* isolates were sampled from multiple pulmonary nodules, one renal lesion and a cavitary pulmonary lesion in the right lung lobe, which also demonstrated voriconazole susceptibility on *in vitro* antifungal susceptibility testing (Kolwijck et al., 2016). A third immunocompromised post-renal transplant patient with IA had a voriconazole-susceptible *A. fumigatus* isolate sampled from BAL and was started on voriconazole, however therapy was stopped after only six days as evidence of IA was deemed to be limited based on galactomannan and CT scan results. However, follow up CT scan confirmed lesions typical of IA, with voriconazole-resistant *A. fumigatus* with the TR_{34}/L98H mutation isolated from sputum sample, and the patient was switched to liposomal amphotericin *B.* Follow-up sputum sampling nine days later cultured voriconazole-susceptible *A. fumigatus* from sputum. Anidulafungin was also added to the treatment regime, however the patient re-presented with delirium and an MRI revealed two brain lesions, with frontotemporal cerebral lobe biopsy showing large B-cell lymphoma and a voriconazole-resistant *A. fumigatus* isolate with TR_{34}/L98H mutation was also cultured from brain biopsy tissue; the patient died two weeks later (Kolwijck et al., 2016). These cases highlight the difficulties in treating aspergillosis in areas where azole resistant *A. fumigatus* are prevalent. The authors suggested that in regions where environmental isolates of *A. fumigatus* have identified *cyp51A* mutations, co-infection with azole-susceptible and azole-resistant isolates should be anticipated and necessitate the close monitoring of IA patients that have recovered azole-susceptible *A. fumigatus* isolates. They also questioned the use of azole monotherapy in regions with the environmental mutations TR_{34}/L98H and TR_{46}/Y121F/T289A (Kolwijck et al., 2016).

The array of available antifungal therapeutics is limited and azole drugs are of vital importance for prophylaxis and treatment of aspergillosis in human patients, making the spread of ARAf throughout the world a major concern for the medical community, and necessitating ongoing surveillance for resistance.

2.3.15 Environmental antifungal use and the development of azole resistance

Antifungal pesticides are used on agricultural crops throughout the world to protect from spoilage and prevent substantial economic losses and shortage of supply. Globally azole fungicides (DMIs) are used for this purpose. These drugs are closely related to the medical triazoles used to treat aspergillosis. European cereal production is heavily reliant on the use of fungicides for agriculture, being one of the biggest users worldwide (Kuck and Russell, 2006).

Environmentally acquired mutations are most commonly reported amongst clinical isolates of *A. fumigatus*, and their presence in the environment has been associated with the use of azole fungicides in agriculture (Snelders et al., 2009, van der Linden et al., 2011, Snelders et al., 2012). Agricultural use of low-cost and broad spectrum azole fungicides is a global practice for food safety and protection of crops against plant fungal pathogens such as rusts and mildews (Price et al., 2015). Azole fungicides, including epoxiconazole, tebuconazole, bromuconazole, difenconazole, propiconazole and metconazole, in use since the 1990s, are structurally similar to those used for medical therapies (Snelders et al., 2009, Snelders et al., 2012). They are the most commonly used agricultural fungicides in Europe (Snelders et al., 2012, Price et al., 2015, Meis et al., 2016) and they have been demonstrated to induce cross-resistance to medical triazole drugs in laboratory tests (Snelders et al., 2009, Snelders et al., 2012). Studies from Europe have demonstrated this agricultural azole fungicide application has led to mutations in the *cyp51A* gene causing azole-resistance in *A. fumigatus* isolates. It has been demonstrated that the TR_{34}/L98H mutation arose in the late-1990s from the agricultural application of azoles, with the allele spreading throughout the world's population of *A. fumigatus* (Snelders et al., 2009, Verweij et al., 2009b, Chowdhary et al., 2013). The ability for *A. fumigatus* isolates with prolonged exposure to agricultural azoles to develop resistance due to *cyp51A* mutations to medical azoles has also been demonstrated in the laboratory setting (Ren et al., 2017).

ARAf is thought to develop through the long-term use of azole therapy leading to selection pressure for azole resistant *A. fumigatus*, or from environmentally acquired azole resistant

strains of *A. fumigatus* (Stensvold et al., 2012). The TR_{34}/L98H mechanism is the most commonly reported cause of ARAf, being found in both azole-naïve and azole-exposed patients. For this reason this mode of resistance is thought to be environmentally acquired. The development and spread of environmentally acquired ARAf has been linked to the use of DMI fungicides for crop protection. Five commonly used DMIs have been demonstrated to have similar mechanisms of action against *A. fumigatus* as medical triazoles - epoxiconazole, bromuconazole, propiconazole, tebuconazole and difenconazole. All these DMIs were in use in the Netherlands prior to the report of the first TR_{34}/L98H mutation in the late 1990s (Snelders et al., 2009, Snelders et al., 2012). Other *cyp51A* resistance mechanisms that are thought to be environmentally acquired include the TR_{46}/Y121F/T289A and G54 (van der Linden et al., 2013, Sharma et al., 2015). An investigation into the exposure concentrations for DMIs in their various applications in the health, agricultural and materials industries demonstrated the highest exposure concentrations occur during medical therapy and treatments applied to wood, certain fruits and seeds, with greatly lowered exposure rates (up to 100 times lower) when DMIs are applied to crops (Gisi, 2014). Thus on the basis that medical treatment is prolonged and fruit and seed applications tend to only occur once per season, the study concluded the likeliest source of resistance development is medical treatments (Gisi, 2014). However for azole-naïve patients, environmental origin of ARAf is more plausible.

Environmental ARAf have been isolated in Europe, Asia, North and South America and Africa, with a prevalence of between 0 – 91%, and the majority of reported mutations due to TR_{34}/L98H (Table 4). Studies that have screened for ARAf in the environment have investigated air, soil, organic debris and composts (Snelders et al., 2009, Mortensen et al., 2010, Chowdhary et al., 2012b, Badali et al., 2013, van der Linden et al., 2013, Chowdhary et al., 2014b, Prigitano et al., 2014, Chowdhary et al., 2014a, Ahmad et al., 2014, Sharma et al., 2015, Bader et al., 2015, Kano et al., 2015, Le Pape et al., 2016, Toyotome et al., 2016, Jeanvoine et al., 2017, Zhang et al., 2017, Hurst et al., 2017, Tangwattanachuleeporn et al., 2017, Ren et al., 2017, Santoro et al., 2017). Environmental investigations from the Netherlands have demonstrated a high prevalence of ARAf in the environment. One study reported a 7% (20/297 environmental indoor and outdoor isolates) prevalence of itraconazole resistance, and the TR_{34}/L98H mechanism was found in 86% of isolates that underwent *cyp51A* sequencing (13/15 isolates) (Snelders et al., 2009). Microsatellite genotyping of these isolates found they clustered with Dutch clinical ARAf strains harbouring the same mutation (TR_{34}/L98H). Clinical and environmental isolates were also tested for resistance to DMIs, demonstrating tebuconazole

and metconazole resistance in itraconazole resistant isolates (Snelders et al., 2009). Another study screening for azole resistant isolates found a total of 140 ARAf isolates from indoor and outdoor air samples taken at hospitals and patient domiciles, with 90% (126) of these harbouring the TR_{34}/L98H mechanism and 10% (14) the TR_{46}/Y121F/T289A mechanism (van der Linden et al., 2013). Another study found a high prevalence of ARAf in composts treated with azoles (91%, 41/45 isolates) compared to azole free compost (2%, 1/50 isolates) (Zhang et al., 2017). Of these ARAf compost isolates, 80% had the *cyp51A* mechanism TR_{46}/Y121F/T289A, 2% had TR_{46}/Y121F/M172I/T289A/G448S and 9% had a novel mechanism associated with pan-azole resistance, TR_{46}^{3}/Y121F/M172I/T289A/G448S (Zhang et al., 2017).

A French study investigating the prevalence of ARAf in 20 French sawmills, found a 3% prevalence in substrate samples (20/600 samples) (Jeanvoine et al., 2017). A total of 24 ARAf strains were isolated, with 83% (20/24) harbouring the TR_{34}/L98H mechanism, two strains with TR34/L98H/S297T/F495I, one with P216L and one with no mutations or promoter region changes. Seventeen of 24 ARAf isolates were taken from eight sawmills that used DMIs propiconazole and tebuconazole to treat wood via dipping in azole tanks, with 14/17 of these isolates sampled from tank areas (Jeanvoine et al., 2017). 88% (21/24 isolates) were pan-azole resistant to itraconazole, voriconazole, posaconazole and isavuconazole, and were also resistant to tebuconazole and propiconazole. One strain had itraconazole resistance only, another itraconazole and posaconazole resistant, and one had resistance to itraconazole, posaconazole and isavuconazole with intermediate voriconazole susceptibility. The authors also found a significant increase in ARAf isolates from sawmills that used both propiconazole and tebuconazole DMIs (Jeanvoine et al., 2017).

An Italian study investigating the presence of ARAf in environmental soil isolates from vineyards, gardens and agricultural crops found a high prevalence of 13%, with 6/47 samples growing ARAf isolates (Prigitano et al., 2014). Nine ARAf isolates were obtained and all had high MICs for itraconazole and posaconazole, and seven isolates also had elevated MICs for voriconazole. *Cyp51A* sequencing revealed seven isolates carried the TR_{34}/L98H mechanism, one isolate had a number of single point mutations including F46Y/M172V/N248T/D255E and the remaining isolate had no mutations or changes to the promoter region of the *cyp51A* gene (Prigitano et al., 2014). Another study examining environmental isolates from Italy, Spain and Hungary found no ARAf in compost and silage samples tested for susceptibility to the DMIs

epoxiconazole and difenconazole, the medical triazole posaconazole and the veterinary azole enilconazole (Santoro et al., 2017). Only one isolate from green compost in Hungary showed reduced susceptibility to posaconazole, and no *cyp51A* mutations commonly associated with azole resistance were found amongst 15 isolates tested (Santoro et al., 2017).

An Indian study investigating environmental soil and air isolates found a prevalence of ARAf of 5% (24/486 samples) (Chowdhary et al., 2012b). Resistant isolates grew from soil from tea gardens, cotton trees, rice paddy fields, hospital garden flower pots, soil containing bird droppings and air from hospital ward. Isolates demonstrated pan-azole resistance and all had the TR_{34}/L98H gene, and were demonstrated to be genetically identical on microsatellite testing (Chowdhary et al., 2012b). A study from Thailand looking at azole resistance in agricultural environmental isolates of *A. fumigatus* found a prevalence of 3.3% (10/308) (Tangwattanachuleeporn et al., 2017). All the isolates were resistant to itraconazole, with 8/10 isolates carrying the TR_{34}/L98H Cyp51A mutation and two isolates also showed posaconazole resistance and carried the G54R mutation, while one isolate also had resistance to voriconazole (Tangwattanachuleeporn et al., 2017). An Iranian study investigating ARAf in soil from hospital surroundings found a prevalence rate of 3.3%, and all carried the TR_{34}/L98H mutation (Badali et al., 2013). The isolates were found to be related to Dutch and Indian clinical and environmental ARAf isolates that also harboured the TR_{34}/L98H mechanism (Badali et al., 2013). Interestingly, a study from Japan investigating air samples from a pumpkin farm where tetraconazole was used twice a year for 15 years found a prevalence of ARAf isolates of 0% (Kano et al., 2015), and a second study from Japan investigating soil isolates from farms where tetraconazole and tebuconazole were used also found an ARAf prevalence of 0% (Toyotome et al., 2016).

A recent study from China found an ARAf prevalence of 4.1% (3/73 isolates) in greenhouse agricultural soil (Ren et al., 2017). Two of the resistant isolates demonstrated voriconazole resistance and had the TR_{46}/Y121F/T289A mechanism, and one isolate with itraconazole resistance had the TR_{34}/L98H/S297T/F495I mechanism (Ren et al., 2017). A Tanzanian study found ARAf in 13.9% of environmental isolates of soil and woody debris from the grounds of a medical centre, with 11/15 isolates carrying the TR_{34}/L98H mutation (pan-azole resistance) and 4/15 the TR_{46}/Y121F/T289A mutation (high MICs of voriconazole and isavuconazole) (Chowdhary et al., 2014b). Interestingly the genotype for the TR_{46}/Y121F/T289A strains was identical to Dutch clinical isolates harbouring the same mutation, while the TR_{34}/L98H

mutation was identical to a clinical isolate from India (Chowdhary et al., 2014b). More recently tulip plant bulbs imported from the Netherlands to Ireland were noted to harbour ARAf isolates, and ARAf isolates with the TR_{34}/L98H mutation and the TR_{46}/Y121F/T289A mutation were also found in the environment (soil, compost and air) in Ireland (Dunne et al., 2017), and as mentioned above, at the home of a patient with ARAf in France (Lavergne et al., 2017). The TR_{34}/L98H mutation has also been isolated from compost of azole sprayed peanut crops in the USA (Hurst et al., 2017). In France ARAf with the mutation TR_{34}/L98H has been isolated from the environment of sawmills, with a significant link made between the number of ARAf strains and the quantity of propiconazole fungicide present (Jeanvoine et al., 2017). A Danish study reported an ARAf prevalence of 11% (4/38 *A. fumigatus* isolates) in soil samples from hospital surroundings and flowerbeds, all harbouring the TR_{34}/L98H mechanism (Mortensen et al., 2010). The same study also investigated soil samples from Spain and Austria, with no ARAf found (Mortensen et al., 2010).

In a letter to the Editor, a research group screening for ARAf on itraconazole or voriconazole supplemented agar amongst soil samples from flower fields and greenhouses in Colombia identified 20 *A. fumigatus* resistant strains (Le Pape et al., 2016). *Cyp51A* sequencing revealed diverse genotypic resistance mechanisms, including TR_{34}/L98H (n=1), TR_{46}/Y121F/T289A (n=17) and TR_{53} (n=1), while one isolate had wild-type *cyp51A* (Le Pape et al., 2016). A more recent study published by the same group investigating itraconazole and voriconazole resistance in soil isolates of *A. fumigatus* from Colombian flower fields and flower beds found a prevalence of ARAf of 9.3% (8/86 soil samples) and the resistance mechanisms TR_{46}/Y121F/T289A (associated with itraconazole and voriconazole resistance), TR_{34}/L98H and TR_{53} (associated with itraconazole resistance only) were also found. Soil samples were examined for fungicide residues and the DMIs tetraconazole, penconazole, tebuconazole and difenconazole were found in flower crop soil, with no residues identified in public gardens soil samples (Alvarez-Moreno et al., 2017).

Single point mutations in G54 leading to amino acid changes have been associated with resistance to itraconazole and posaconazole, and were originally thought to develop in patients on azole therapy. However a recent study reported this mutation (G54E) in environmental isolates collected in India (20% of ARAf isolates tested), Tanzania (46.4% of ARAf isolates tested) and Romania (30.4% of ARAf isolates tested) (Sharma et al., 2015). These isolates demonstrated resistance to itraconazole, posaconazole and the agricultural fungicides

penconazole, bromuconazole, difenconazole, epoxiconazole, cyproconazole, triadimefon, tricyclazole, tebuconazole and hexaconazole. They were susceptible to voriconazole, isavuconazole and the fungicide metconazole. Genotype analysis showed most G54E resistant isolates from Romania and Tanzania were identical, and all Tanzanian isolates were identical. These were distinct from Dutch clinical isolates harbouring this mutation (Sharma et al., 2015). The one Indian isolate with the G54E mutation demonstrated a distinct genotype to all other isolates examined. The authors of this study suggested long term exposure to agricultural fungicides likely led to selection for resistant phenotype and genotype in the environment. This study found an overall prevalence of ARAf in Tanzania, Romania and India of 26.4% (28/106), 24.2% (23/95) and 9.6% (5/52) respectively (Sharma et al., 2015).

A study on environmental isolates from indoor and outdoor hospital areas in Kuwait found pan-azole resistance to itraconazole, voriconazole and posaconazole in 8/115 *A. fumigatus* isolates (7%) (Ahmad et al., 2014). All isolates harboured the TR_{34}/L98H mechanism and microsatellite typing revealed three distinct groups of isolates, one group clustering with clinical and environmental isolates from India. There was no clustering of Kuwait isolates with Iranian, Asian or European isolates (Ahmad et al., 2014). Another Kuwait study examining triazole susceptibility (itraconazole, posaconazole and voriconazole) of environmental (n=50) and clinical (n=16) *Aspergillus fumigatus* isolates from single colonies demonstrated a mix of azole resistance and azole susceptible strains in serial dilutions of single colonies form three isolates (1 environmental, 2 clinical) (Ahmad et al., 2015). All resistant strains carried the TR_{34}/L98H mutation. This demonstrated that *A. fumigatus* colonies could potentially be made up of different conidia (Ahmad et al., 2015).

Another tandem repeat in the *cyp51A* gene of 53 base pairs has been identified in an *A. fumigatus* isolate from a patient with chronic granulomatous disease receiving itraconazole prophylaxis (Hodiamont et al., 2009). This patient developed osteomyelitis due to *A. fumigatus*, and the recovered isolate was resistant to itraconazole and voriconazole, with reduced susceptibility to posaconazole (Hodiamont et al., 2009). This 53bp mutation has also been found in Colombian environmental isolates of *A. fumigatus* that were resistant to itraconazole (Le Pape et al., 2016, Alvarez-Moreno et al., 2017).

To date there have been no environmental prevalence investigations for ARAf in Australia.

The apparent migration of ARAf from the Netherlands throughout Europe and into Asia and Africa is a public health concern that should be monitored globally.

Table 4. Summary of sample and isolate details from environmental prevalence studies investigating azole resistant *Aspergillus fumigatus*.

Study	**Country**	**Substrate**	**No. samples tested**	**No. samples yielding AF (%)**	**No. samples yielding ARAf/Total samples tested (%)**	**No. ARAf isolates/Total AF isolates (%)**
Snelders et al., 2009	The Netherlands	Soil, compost, seeds, leaves	79	49/79 (62%)	10/49 (20%)	NS
Snelders et al., 2009	The Netherlands	Hospital indoor air and water	248	248/248 (100%)	5/248 (2%)	5/248 (2%)
van der Linden et al., 2013	The Netherlands	Air from hospitals and domiciles	21	NS	18/21 (86%)	NS
Zhang et al., 2017	The Netherlands	Azole-exposed compost	6	6/6 (100%)	NS	41/45 (91%)
Zhang et al., 2017	The Netherlands	Azole-naive compost	6	6/6 (100%)	NS	1/50 (2%)
Mortensen et al., 2010	Austria	Soil, compost	50	144/185 (78%)	0/49 (0%)	0
Mortensen et al., 2010	Denmark	Soil, compost	76		4/76 (5%)	4/55 (7%)
Mortensen et al., 2010	Spain	Soil, compost	59		0/59 (0%)	0
Bader et al., 2015	Germany	Soil	455	NS	NS	55/452 (12%)
Jeanvoine et al., 2017	France	Air in 20 sawmills	200	91/200 (46%)	0/200 (0%)	0
Jeanvoine et al., 2017	France	Sawmill substrates in 20 sawmills	600	279/600 (47%)	20/600 (3%)	24/150 (16%)
Prigitano et al., 2014	Italy	Soil from vineyards, agricultural crops, gardens, compost from garden centre and flower pots	47	29/47 (62%)	6/47 (12.8%)	NS

Santoro et al., 2017	Italy	Compost	56	NS	NS	0/5 (0%)
Santoro e et al., 2017	Spain	Compost	56	NS	NS	0/20 (0%)
Santoro et al., 2017	Hungary	Compost	56	NS	NS	0/8 (0%)
Badali et al., 2013	Iran	Garden soil	150	NS	5/150 (3%)	5/41 (12%)
Ahmad et al., 2014	Kuwait	Air, water and cotton swabs from indoor/outdoor environments, soil	362	115/362 (32%)	NS	8/115 (7%)
Ahmad et al., 2015	Kuwait	Soil sample, hospital door swabs	50	50/50 (100%)	1/50 (2%)	1/50 (2%)
Chowdhary et al., 2014	Tanzania	Soil, woody debris	30	29/30 (97%)	6/30 (20%)	15/108 (14%)
Sharma et al., 2015	Tanzania	Soil, woody debris	30	73/81 (90%)	10/30 (33%)	28/106 (26%)
Sharma et al., 2015	Romania	Soil, woody debris	27		5/27 (19%)	23/95 (24%)
Sharma et al., 2015	India	Soil, woody debris	24		5/24 (21%)	5/52 (9.6%)
Chowdhary et al., 2012	India	Soil from crops, garden beds, flower pots, hospital gardens, containing bird droppings, air from hospital ward	486	201/486 (41%)	24/486 (5%)	44/630 (7%)
Chowdhary et al., 2014	India	Soil from agricultural crop and flower beds	105	37/105 (35%)	NS	14/126 (11%)
Kano et al., 2015	Japan	Air from azole-treated pumpkin farm	50	NS	0/50 (0%)	0/50 (0%)
Toyotome et al., 2016	Japan	Soil from azole-treated farms	11	11/11 (100%)	0/11 (0%)	0/91 (0%)
Tangwattan-achuleeporn et al., 2017	Thailand	Soil	308	99/308 (32%)	10/308 (3%)	10/99 (10%)
Ren et al., 2017	China	Greenhouse soil	144	64/144 (44%)	3/144 (2%)	3/73 (4%)

Le Pape et al., 2016[*]	Colombia	Soil, flower fields and greenhouses	38	20/38 (53%)	20/20 (100%)[#]	NS
Hurst et al., 2017	USA	Azole-treated peanut crop debris, soil	34	8/34 (24%)	NS	38/200 (19%)

Abbreviations: ARAf, azole resistant *Aspergillus* fumigatus; AF *Aspergillus fumigatus*; NS not stated. [*]Letter to the Editor; [#]selected resistant *A. fumigatus* isolates for the study.

2.3.16 Rapid diagnosis of azole-resistant *A. fumigatus* strains in the clinical setting

More rapid methods of screening for azole resistance in the clinical setting are being introduced to identify resistant cases faster, including azole screening tests and molecular screening tests targeting *cyp51A* mutations that are well-known to confer azole resistance.

In recent years the VIPcheck™ (AMT Plates (Balis Laboratorium V.O.F., Boven Leeuwen, the Netherlands) testing kit has been developed commercially in the Netherlands and demonstrated to be sensitive and specific for quick in-house screening of isolates against the most commonly used antifungal azole drugs itraconazole, voriconazole and posaconazole (van der Linden et al., 2009, van der Linden et al., 2013, van der Linden et al., 2015). The plate has four agar wells, with three containing itraconazole, voriconazole and posaconazole and one antifungal-free growth control well. Clinical testing of the kit has been performed as part of a national surveillance program in the Netherlands, where *A. fumigatus* isolates were screened for resistance using the kit and any positive isolates were sent for further testing at the National Mycology Reference Laboratory for further confirmation with microbroth dilution antifungal susceptibility testing and *cyp51A* gene sequencing (Lestrade et al., 2016). The test has been demonstrated to be a valuable screening test, with a reported sensitivity and specificity of 99% for detecting ARAf due to TR_{34}/L98H, G54, M220 and TR_{46}/Y121F/T289A (van der Linden et al., 2013, Lestrade et al., 2016, Arendrup et al., 2017b). The screening plate has the potential benefits of early ARAf detection for more appropriate therapeutic selection and increased ARAf screening made possible by this easy to use test could help uncover the true epidemiology of ARAf (Arendrup et al., 2017b).

Targeted PCR assays have been developed to identify specific resistance associated mutations in *A. fumigatus* samples, including the resistance mechanisms TR_{34}/L98H, and single point mutations. Molecular screening for ARAf was first successfully performed with a novel mixed-

format real-time PCR assay looking for the specific single point mutations L98, G54, M220 and G138 and the promoter region (Klaassen et al., 2010). A total of 209 clinically relevant *A. fumigatus* isolates were tested for ARAf using the CLSI M38-A2 method. Four isolates demonstrated multi-azole resistance to itraconazole, voriconazole, posaconazole and isavuconazole. The TR_{34}/L98H resistance mechanism was identified in all four resistant isolates in this study. This study also determined a low ARAf prevalence rate of 1.9% (4/209) amongst patients in the Netherlands (Klaassen et al., 2010). Another study developed a rapid real-time PCR method for detection of G54 mutations conferring resistance in *A. fumigatus* isolates (Balashov et al., 2005).

Molecular technology has made screening for ARAf without a positive culture possible. A high prevalence of *cyp51A* mutations (55.1%) were found in sputum samples from patients with chronic fungal disease that were culture negative but had *Aspergillus* DNA on PCR (Denning et al., 2011a). TR_{34}/L98H was detected in 75% (6/8) of patients with ABPA and the M220K (n=2) and M220R (n=2) mutations were detected in 50% (12/24) of patients with CPA (Denning et al., 2011a). More recently the AsperGenius® (PathoNostics, Maastricht, NL) assay has been demonstrated to be useful for identifying common azole mutations (TR_{34}/L98H, T289A, Y121F) and *Aspergillus* species in BAL fluid (sensitivity 80% to 88.9%, specificity 80% to 93.3%, positive predictive value 72.7% to 80%, negative predictive value 87% to 93.3%), serum (sensitivity 78.6%, specificity 100%) and with varying reliability in plasma (sensitivity 80% to 100%, specificity 50 to 77.8%) (Chong et al., 2015, Chong et al., 2016, White et al., 2017, Chong et al., 2017, Montesinos et al., 2017, Schauwvlieghe et al., 2017).

2.3.17 Azole-resistant *A. fumigatus* in veterinary patients

There are few reports of *A. fumigatus* triazole resistance in the veterinary literature (Table 5). Studies on samples from domestic and wild birds and their environments in Europe and Asia have reported a resistance prevalence of 0 to 35.7% (Beernaert et al., 2009, Wang et al., 2014), however not all studies have investigated resistance mechanisms. One study found azole-resistant phenotypes amongst birds previously treated with itraconazole, however no *cyp51A* sequencing was performed (Beernaert et al., 2009). Another study looking at samples from poultry farms in France, where the imidazole parconazole had been used prophylactically, and China, where the poultry farm was azole naïve, found no azole resistant isolates. However on *cyp51A* sequencing nine single point mutations were found, including F46Y, M172V, N248T,

N248K, D255E, E427K, A116R, E130D, Q131H (Wang et al., 2014). A Polish study using the CLSI M44 disk diffusion and M38-A2 microbroth dilution testing standards found a high prevalence of 14.1% itraconazole resistance in *A. fumigatus* isolates form domestic geese, however *cyp51A* sequence analysis was not performed and no other resistance mechanisms were investigated or determined (Ziolkowska et al., 2014). Interestingly the avian isolates also demonstrated concomitant resistance to imidazoles and amphotericin B (Ziolkowska et al., 2014).

Table 5. Summary of veterinary studies investigating prevalence of azole-resistant strains of *A. fumigatus*

Country (Publication)	**Animal Species**	**Sample site**	**No. *A. fumigatus* isolates**	**Antifungal susceptibility testing method**	**ARAf prevalence**	***Cyp51A* alterations**
Belgium, the Netherlands (Beernaert et al., 2009)	Domestic and wild avian species	Not specified	59	Broth micro-dilution (CLSI M38-A2)	6.8%	Not tested
France, China (Wang et al., 2014)	Domestic Guinea fowl, turkeys and chickens, other avian species	Pharyngeal swabs, laboratory collection isolates from birds with aspergillosis	175 (124 France, 51 China)	Etest (BioMerieux, Lyon, France), CLSI M38-A2	0%	F46Y M172V N248T N248K D255E E427K A116R E130D Q131H
Poland (Ziolkowska et al., 2014)	Domestic geese	Oral swabs, environment	85	Disk diffusion (CLSI M44) Broth micro-dilution (CLSI M38-A2)	14.1%	Not tested

2.3.18 *Cyp51A* homology modelling

A. fumigatus cyp51A homology models have explored the impact of amino acid changes (mutations) on azole drug interaction with the conserved *cyp51A* target protein site. As the

crystal structure of the *A. fumigatus cyp51A* gene has not yet been characterised, existing homology models have based on c*yp51A* crystal structures of other species, including bacteria (*M. tuberculosis)*, humans (*H. sapiens*) and non-*Aspergillus* fungi (*S. cerevisiae*), all with varying percentage similarities to *A. fumigatus cyp51A*. These models offer predictions of the significance of *A. fumigatus* mutations (Lewis et al., 1999, Xiao et al., 2004, Zhao et al., 2007, Sheng et al., 2009, Snelders et al., 2010, Fraczek et al., 2011, Liu et al., 2016). The recent modelling of the *A. fumigatus* c*yp51B* gene, a paralogue gene of c*yp51A* that also conserves metabolic roles of 14-alpha-demethylase, demonstrated the closest percentage similarity with wild-type *A. fumigatus cyp51A* to date (Hargrove et al., 2015, Nash and Rhodes, 2017).

Homology modelling has shown there are two ligand access channels to the active site of the *cyp51A* protein (the haem centre), and it is thought azole drugs utilise these channels to gain access to the target site (Snelders et al., 2010). Therefore mutations occurring close to the target protein have been determined to be more likely to affect azole docking access, and protein structural stability and amino acid changes occurring near the access channel openings are thought to disrupt azole molecules from docking to the protein, thereby enabling azole resistance (Snelders et al., 2010). Mutations on the less conserved periphery of the protein are less likely to be associated with azole resistance (Snelders et al., 2010).

A study using the *cyp51A* crystal structure of *M. tuberculosis* (a 28% similarity to *A. fumigatus cyp51A*) concluded that mutations in G54, G138, M220 and L98H were all located close to one of the protein's two ligand access channels and so likely interfered with azole molecule docking (Snelders et al., 2010). Other mutations located peripherally did not change the protein's structural integrity and did not interact with azole drugs, including F46Y, N248T, M172V, L358L, E427K and D255E (Snelders et al., 2010). Another study then used the human *cyp51A* protein to develop a model for *A. fumigatus* (Fraczek et al., 2011). The human *cyp51A* protein had a higher similarity of 41% and demonstrated mutations at G54 and M220 could block binding channel access for the long-chain azoles itraconazole and posaconazole, and G448, positioned adjacent to the haem, could alter its position in the protein. The model could not demonstrate how L98H could alter its structure (Fraczek et al., 2011). A recent study using *cyp51A* modelling based on the fungus *S. cerevisiae* (similarity of 50% with *A. fumigatus cyp51A* protein) found mutations at L98, M220 and Y431 decrease binding affinity of azoles to the protein, and L98 and G432 mutations reduce protein stability (Liu et al., 2016). This study also observed that some mutations associated with azole resistance increased ratherthan

decreased protein model stability, including G54, G138, H147, G432 and F495 (Liu et al., 2016). To date, the *A. fumigatus cyp51B* model with the closest percentage similarity to *A. fumigatus cyp51A* (59%) has only been used to demonstrate how the L98H mutation confers resistance, determining the histidine substitution forms hydrogen bonds with neighbouring polar side chains that decreases the active site surface, potentially interfering with the azole drug binding process (Nash and Rhodes, 2017).

Protein homology modelling offers a prediction of the significance of *A. fumigatus cyp51A* mutations that is limited by its similarity to *cyp51A* in other biological species and potential differences in functionality to other *cyp51* genes in the same species.

2.4 References

ABDO, W., KAWACHI, T., SAKAI, H., FUKUSHI, H., KANO, R., SHIBAHARA, T., SHIROUZU, H., KAKIZOE, Y., TUJI, H. & YANAI, T. 2012. Disseminated mycosis in a killer whale (*Orcinus orca*). *Journal of Veterinary Diagnostic Investigation,* 24**,** 211-8.

ABDOLRASOULI, A., RHODES, J., BEALE, M. A., HAGEN, F., ROGERS, T. R., CHOWDHARY, A., MEIS, J. F., ARMSTRONG-JAMES, D. & FISHER, M. C. 2015. Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. *mBio,* 6**,** e00536.

ABRAMS, G. A., PAUL-MURPHY, J., RAMER, J. C. & MURPHY, C. J. 2001. *Aspergillus* blepharitis and dermatitis in a peregrine falcon-gyrfalcon hybrid (*Falco peregrinus* X *Falco rusticolus*). *Journal of Avian Medicine and Surgery,* 15**,** 114-120.

ADAMAMA-MORAITOU, K. K., PAITAKI, C. G., RALLIS, T. S. & TONTIS, D. 2001. *Aspergillus* species cystitis in a cat. *Journal of Feline Medicine and Surgery,* 3**,** 31-34.

ADAMAMA-MORAITOU, K. K., PARDALI, D., DAY, M. J., DENNING, D. W., PAPAZOGLOU, L., PAPASTEFANOU, A. & RALLIS, T. S. 2011. *Aspergillus fumigatus* bronchopneumonia in a Hellenic shepherd dog. *Journal of the American Animal Hospital Association,* 47**,** E13-E18.

AHMAD, S., JOSEPH, L., HAGEN, F., MEIS, J. F. & KHAN, Z. 2015. Concomitant occurrence of itraconazole-resistant and -susceptible strains of *Aspergillus fumigatus* in routine cultures. *Journal of Antimicrobial Chemotherapy,* 70**,** 412-5.

AHMAD, S., KHAN, Z., HAGEN, F. & MEIS, J. F. 2014. Occurrence of triazole-resistant *Aspergillus fumigatus* with TR34/L98H mutations in outdoor and hospital environment in Kuwait. *Environmental Research,* 133**,** 20-6.

AINSWORTH, G. C. & REWELL, R. E. 1949. The incidence of aspergillosis in captive wild birds. *Journal of Comparative Pathology,* 59**,** 213-224.

AKAN, M., HAZIROĞLU, R., ILHAN, Z., SAREYYÜPOĞLU, B. & TUNCA, R. 2002. A case of aspergillosis in a broiler breeder flock. *Avian Diseases,* 46**,** 497-501.

ALANIO, A., CABARET, O., SITTERLE, E., COSTA, J. M., BRISSE, S., CORDONNIER, C. & BRETAGNE, S. 2012. Azole preexposure affects the *Aspergillus fumigatus* population in patients. *Antimicrobial Agents and Chemotherapy,* 56**,** 4948-50.

ALANIO, A., DENIS, B., HAMANE, S., RAFFOUX, E., PEFFAULT DE LATOUR, R., MENOTTI, J., AMORIM, S., TOURATIER, S. & BERGERON, A. 2016. Azole resistance of *Aspergillus fumigatus* in immunocompromised patients with invasive aspergillosis. *Emerging Infectious Diseases,* 22**,** 157-158.

ALANIO, A., SITTERLÉ, E., LIANCE, M., FARRUGIA, C., FOULET, F., BOTTEREL, F., HICHERI, Y., CORDONNIER, C., COSTA, J. M. & BRETAGNE, S. 2011. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. *Journal of Antimicrobial Chemotherapy,* 66**,** 371-374.

ALASTRUEY-IZQUIERDO, A., ALCAZAR-FUOLI, L. & CUENCA-ESTRELLA, M. 2014. Antifungal susceptibility profile of cryptic species of *Aspergillus*. *Mycopathologia,* 178**,** 427-433.

ALASTRUEY-IZQUIERDO, A., MELHEM, M. S. C. B., L. X. & RODRIGUEZ-TUDELA, J. L. 2015. Susceptibility test for fungi: clinical and laboratorial correlations in medical mycology. *The Revista do Instituto de Medicina Tropical de Sao Paulo,* 27**,** 57-64.

ALASTRUEY-IZQUIERDO, A., MELLADO, E., PELÁEZ, T., PEMÁN, J., ZAPICO, S., ALVAREZ, M., RODRÍGUEZ-TUDELA, J. L., CUENCA-ESTRELLA, M. & AND FILPOP STUDY GROUP 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrobial Agents and Chemotherapy,* 57**,** 3380-3387.

ALCAZAR-FUOLI, L., CUESTA, I., RODRIGUEZ-TUDELA, J. L., CUENCA-ESTRELLA, M., SANGLARD, D. & MELLADO, E. 2011. Three-dimensional models of 14alpha-sterol demethylase (*Cyp51A*) from *Aspergillus lentulus* and *Aspergillus fumigatus*: an insight into differences in voriconazole interaction. *International Journal of Antimicrobial Agents,* 38**,** 426-34.

ALCAZAR-FUOLI, L., MELLADO, E., ALASTRUEY-IZQUIERDO, A., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2008. *Aspergillus* section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. *Antimicrobial Agents and Chemotherapy,* 52**,** 1244-1251.

ALHAMBRA, A., CATALÁN, M., MORAGUES, M. D., BRENA, S., PONTÓN, J., MONTEJO, J. C. & DEL PALACIO, A. 2008. Isolation of *Aspergillus lentulus* in Spain from a critically ill patient with chronic obstructive pulmonary disease. *Revista iberoamericana de micología,* 25**,** 246-249.

ALLIZOND, V., TULLIO, V., CUFFINI, A. M., ROANA, J., SCALAS, D., MARRA, E. S., PIERSIGILLI, G., MERLINO, C., MANDRAS, N. & BANCHE, G. 2016. *Advances in microbiology, infectious diseases and public health: fungal occurrence in the hair and skin of symptomatic pets in Turin, Italy,* Cham, Switzerland, Springer International Publishing AG.

ALVAREZ-MORENO, C., LAVERGNE, R. A., HAGEN, F., MORIO, F., MEIS, J. F. & LE PAPE, P. 2017. Azole-resistant *Aspergillus fumigatus* harboring TR34/L98H, TR46/Y121F/T289A and TR53 mutations related to flower fields in Colombia. *Scientific Reports,* 7**,** 45631.

ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., BLANCO, J. L., GARCIA, M. E., KWON, M., MUÑOZ, P., CUENCA-ESTRELLA, M., BOUZA, E. & PELÁEZ, T. 2014. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. *Journal of Medical Microbiology,* 63**,** 617-619.

ARENDRUP, M. C., VERWEIJ, P. & NIELSEN, H. V. 2017a. Evaluation of MIC strip isavuconazole test for susceptibility testing of wild-type and non-wild-type *Aspergillus fumigatus* isolates. *Antimicrobial Agents and Chemotherapy,* 61.

ARENDRUP, M. C., VERWEIJ, P. E., MOUTON, J. W., LAGROU, K. & MELETIADIS, J. 2017b. Multicentre validation of 4-well azole agar plates as a screening

method for detection of clinically relevant azole-resistant

Aspergillus fumigatus. *Journal of Antimicrobial Chemotherapy,* 72**,** 3325-3333.

ARNE, P., THIERRY, S., WANG, D., DEVILLE, M., LE LOC'H, G., DESOUTTER, A., FEMENIA, F., NIEGUITSILA, A., HUANG, W., CHERMETTE, R. & GUILLOT, J. 2011. *Aspergillus fumigatus* in Poultry. *International Journal of Microbiology,* 2011**,** 746356.

ASTVAD, K. M. T., JENSEN, R. H., HASSAN, T. M., MATHIASEN, E. G., THOMSEN, G. M., PEDERSEN, U. G., CHRISTENSEN, M., HILBERG, O. & ARENDRUP, M. C. 2014. First detection of TR46/Y121F/T289A and TR34/L98H alterations in

Aspergillus fumigatus isolates from azole-naive patients in Denmark

despite negative findings in the environment. *Antimicrobial Agents and Chemotherapy,* 58**,** 5096-5101.

BADALI, H., VAEZI, A., HAGHANI, I., YAZDANPARAST, S. A., HEDAYATI, M. T., MOUSAVI, B., ANSARI, S., HAGEN, F., MEIS, J. F. & CHOWDHARY, A. 2013. Environmental study of azole-resistant *Aspergillus fumigatus* with TR34/L98H mutations in the cyp51A gene in Iran. *Mycoses,* 56**,** 659-63.

BADER, O., TUNNERMANN, J., DUDAKOVA, A., TANGWATTANACHULEEPORN, M., WEIG, M. & GROSS, U. 2015. Environmental isolates of azole-resistant *Aspergillus fumigatus* in Germany. *Antimicrobial Agents and Chemotherapy*, 59**,** 4356-9.

BADER, O., WEIG, M., REICHARD, U., LUGERT, R., KUHNS, M., CHRISTNER, M., HELD, J., PETER, S., SCHUMACHER, U., BUCHHEIDT, D., TINTELNOT, K., GROß, U. & PARTNERS., M.-D. 2013. *cyp51A*-Based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. *Antimicrobial Agents and Chemotherapy,* 57**,** 3513-3517.

BALAJEE, S. A., BORMAN, A. M., BRANDT, M. E., CANO, J., CUENCA-ESTRELLA, M., DANNAOUI, E., GUARRO, J., HAASE, G., KIBBLER, C. C., MEYER, W., O'DONNELL, K., PETTI, C. A., RODRIGUEZ-TUDELA, J. L., SUTTON, D., VELEGRAKI, A. & WICKES, B. L. 2009a. Sequence-based identification of *Aspergillus, Fusarium,* and *Mucorales* species in the clinical mycology laboratory: where are we and where should we go from here? *Journal of Clinical Microbiology,* 47**,** 877-84.

BALAJEE, S. A., GRIBSKOV, J., BRANDT, M., ITO, J., FOTHERGILL, A. & MARR, K. A. 2005a. Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. *Journal of Clinical Microbiology,* 43**,** 5996-5999.

BALAJEE, S. A., GRIBSKOV, J. L., HANLEY, E., NICKLE, D. & MARR, K. A. 2005b. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. *Eukaryotic Cell,* 4**,** 625-632.

BALAJEE, S. A., HOUBRAKEN, J., VERWEIJ, P. E., HONG, S. B., YAGHUCHI, T., VARGA, J. & SAMSON, R. A. 2007. *Aspergillus* species identification in the clinical setting. *Studies in Mycology,* 59**,** 39-46.

BALAJEE, S. A., KANO, R., BADDLEY, J. W., MOSER, S. A., MARR, K. A., ALEXANDER, B. D., ANDES, D., KONTOYIANNIS, D. P., PERRONE, G., PETERSON, S., BRANDT, M. E., PAPPAS, P. G. & CHILLER, T. 2009b. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *Journal of Clinical Microbiology,* 47**,** 3138-3141.

BALAJEE, S. A., NICKLE, D., VARGA, J. & MARR, K. A. 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryotic Cell,* 5**,** 1705-1712.

BALASHOV, S. V., GARDINER, R., PARK, S. & PERLIN, D. S. 2005. Rapid, high-throughput, multiplex, real-time PCR for identification of mutations in the *cyp51A* gene of *Aspergillus fumigatus* that confer resistance to itraconazole. *Journal of Clinical Microbiology,* 43**,** 214-22.

BALSEIRO, A., ESPI, A., MARQUEZ, I., PEREZ, V., FERRERAS, M. C., MARIN, J. F. & PRIETO, J. M. 2005. Pathological features in marine birds affected by the prestige's oil spill in the north of Spain. *Journal of Wildlife Diseases,* 41**,** 371-8.

BARACHETTI, L., MORTELLARO, C. M., DI GIANCAMILLO, M., GIUDICE, C., MARTINO, P., TRAVETTI, O. & MILLER, P. E. 2009. Bilateral orbital and nasal aspergillosis in a cat. *Veterinary Opthalmology,* 12**,** 176-182.

BARLEY, J., FOSTER, G., REID, B., DAGLEISH, M. & HOWIE, F. 2007. Encephalitis in a northern bottlenose whale. *The Veterinary Record,* 160**,** 452.

BARNETT, J., BOOTH, P., ARROW, M., GARCIA-RUEDA, C. & IRVINE, R. M. 2011. Spinal aspergillosis in pheasants. *The Veterinary Record,* 169**,** 449-50.

BARRS, V. R., BEATTY, J. A., DHAND, N. K., TALBOT, J. J., BELL, E., ABRAHAM, L. A., CHAPMAN, P., BENNETT, S., VAN DOORN, T. M. & MAKARA, M. 2014. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. *The Veterinary Journal,* 201**,** 215-222.

BARRS, V. R., BEATTY, J. A., LINGARD, A. E., MALIK, R., KROCKENBERGER, M. B., MARTIN, P., O'BRIEN, C., ANGLES, J. M., DOWDEN, M. & HALLIDAY, C. 2007. Feline sino-orbital aspergillosis:an emerging clinical syndrome. *Australian Veterinary Journal,* 85**,** N23.

BARRS, V. R., HALLIDAY, C., MARTIN, P., WILSON, B., KROCKENBERGER, M., GUNEW, M., BENNETT, S., KOEHLMEYER, E., THOMPSON, A., FLIEGNER, R., HOCKING, A., SLEIMAN, S., O'BRIEN, C. & BEATTY, J. A. 2012. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *The Veterinary Journal,* 191**,** 58-64.

BARRS, V. R. & TALBOT, J. J. 2014. Feline aspergillosis. *Veterinary Clinics of North America: Small Animal Practice,* 44**,** 51-73.

BARRS, V. R., UJVARI, B., DHAND, N. K., PETERS, I. R., TALBOT, J. J., JOHNSON, L. R., BILLEN, F., MARTIN, P., BEATTY, J. A. & BELOV, K. 2015. Detection of *Aspergillus*-specific antibodies by agar gel double immunodiffusion and IgG ELISA in feline upper respiratory tract aspergillosis. *The Veterinary Journal,* 203**,** 285-289.

BARRS, V. R., VAN DOORN, T. M., HOUBRAKEN, J., KIDD, S. E., MARTIN, P., PINHEIRO, M. D., RICHARDSON, M., VARGA, J. & SAMSON, R. A. 2013. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One,* 8**,** e64871.

BARTON, R. C., HOBSON, R. P., DENTON, M., PECKHAM, D., BROWNLEE, K., CONWAY, S. & KERR, M. A. 2008. Serologic diagnosis of allergic bronchopulmonary aspergillosis in patients with cystic fibrosis through the detection of immunoglobulin G to *Aspergillus fumigatus*. *Diagnostic microbiology and infectious disease,* 62**,** 287-91.

BECKMAN, B. J., HOWE, C. W., TRAMPEL, D. W., DEBEY, M. C., RICHARD, J. L. & NIYO, Y. 1994. *Aspergillus fumigatus* keratitis with intraocular invasion in I5-day-old chicks. *Avian Diseases,* 38**,** 660-665.

BEERNAERT, L. A., PASMANS, F., VAN WAEYENBERGHE, L., DORRESTEIN, G. M., VERSTAPPEN, F., VERCAMMEN, F., HAESEBROUCK, F. & MARTEL, A. 2009. Avian *Aspergillus fumigatus* strains resistant to both itraconazole and voriconazole. *Antimicrobial Agents and Chemotherapy,* 53**,** 2199-201.

BELLETE, B., RABERIN, H., MOREL, J., FLORI, P., HAFID, J. & MANHSUNG, R. T. 2010. Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Medical Mycology,* 48**,** 197-200.

BILLEN, F., CLERCX, C., LE GARÉRRÈS, A., MASSART, L., MIGNON, B. & PEETERS, D. 2009a. Effect of sampling method and incubation temperature on fungal culture in canine sinonasal aspergillosis. *The Journal of Small Animal Practice,* 50**,** 67-72.

BILLEN, F., GUIEU, L.-V., BERNAERTS, F., MERCIER, E., LAVOUÉ, R., TUAL, C., PEETERS, D. & CLERCX, C. 2010. Efficacy of intrasinusal administration of bifonazole cream alone or in combination with enilconazole irrigation in canine sino-nasal aspergillosis: 17 cases. *The Canadian Veterinary Journal,* 51**,** 164-168.

BILLEN, F., PEETERS, D., PETERS, I. R., HELPS, C. R., HUYNEN, P., DE MOL, P., MASSART, L., DAY, M. J. & CLERCX, C. 2009b. Comparison of the value of measurement of serum galactomannan and *Aspergillus*-specific antibodies in the diagnosis of canine sino-nasal aspergillosis. *Veterinary Microbiology,* 133**,** 358-365.

BITAR, D., LORTHOLARY, O., LE STRAT, Y., NICOLAU, J., COIGNARD, B., TATTEVIN, P., CHE, D. & DROMER, F. 2014. Population-based analysis of invasive fungal infections, France, 2001-2010. *Emerging Infectious Diseases,* 20**,** 1149-55.

BODEY, G. P. & VARTIVARIAN, S. 1989. Aspergillosis. *European Journal of Clinical Microbiology and Infectious Diseases,* 8**,** 413-37.

BOLTON, G. R. & BROWN, T. T. 1972. Mycotic colitis in a cat. *Veterinary Medicine, Small Animal Clinician,* 67**,** 978-981.

BRYANT, B. R., CAMPBELL, M. & SANGSTER, C. 2016. Acute hepatic necrosis and death in a subadult southern white rhinoceros (*Ceratotherium simum*) associated with exposure to sterigmatocystin in forage contaminated with *Aspergillus nidulans*. *Australian Veterinary Journal,* 94**,** 433-434.

BUEID, A., HOWARD, S. J., MOORE, C. B., RICHARDSON, M. D., HARRISON, E., BOWYER, P. & DENNING, D. W. 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *Journal of Antimicrobial Chemotherapy,* 65**,** 2116-8.

BUENVIAJE, G. N., LADDS, P. W., MELVILLE, L. & MANOLIS, S. C. 1994. Disease-husbandry associations in farmed crocodiles in Queensland and the Northern Territory. *Australian Veterinary Journal,* 71**,** 165-73.

BUIED, A., MOORE, C. B., DENNING, D. W. & BOWYER, P. 2013. High-level expression of cyp51B in azole-resistant clinical *Aspergillus fumigatus* isolates. *Journal of Antimicrobial Chemotherapy,* 68**,** 512-4.

BURCO, J. D., ETIENNE, K. A., MASSEY, J. G., ZICCARDI, M. H. & BALAJEE, S. A. 2012. Molecular sub-typing suggests that the environment of rehabilitation centers may be a potential source of *Aspergillus fumigatus* infecting rehabilitating seabirds. *Medical Mycology,* 50**,** 91-8.

BURGEL, P. R., BAIXENCH, M. T., AMSELLEM, M., AUDUREAU, E., CHAPRON, J., KANAAN, R., HONORE, I., DUPOUY-CAMET, J., DUSSER, D., KLAASSEN, C. H., MEIS, J. F., HUBERT, D. & PAUGAM, A. 2012. High prevalence of azole-resistant *Aspergillus fumigatus* in adults with cystic fibrosis exposed to itraconazole. *Antimicrobial Agents and Chemotherapy,* 56**,** 869-74.

BURK, R. L., JOSEPH, R. & BAER, K. 1990. Systemic aspergillosis in a cat. *Veterinary Radiology and Ultrasound,* 31**,** 26-28.

BURR, E. W. 1981. Enzootic aspergillosis in wild red vented cockatoos in the Philippines. *Mycopathologia* 73**,** 21-28.

CACCIUTTOLO, E., ROSSI, G., NARDONI, S., LEGROTTAGLIE, R. & MANI, P. 2009. Anatomopathological aspects of avian aspergillosis. *Veterinary Research Communications,* 33**,** 521-7.

CAMPS, S. M., RIJS, A. J., KLAASSEN, C. H., MEIS, J. F., O'GORMAN, C. M., DYER, P. S., MELCHERS, W. J. & VERWEIJ, P. E. 2012a. Molecular epidemiology of *Aspergillus fumigatus* isolates harboring the TR34/L98H azole resistance mechanism. *Journal of Clinical Microbiology,* 50**,** 2674-80.

CAMPS, S. M., VAN DER LINDEN, J. W., LI, Y., KUIJPER, E. J., VAN DISSEL, J. T., VERWEIJ, P. E. & MELCHERS, W. J. 2012b. Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: a case study and review of the literature. *Antimicrobial Agents and Chemotherapy,* 56**,** 10-6.

CAMPS, S. M. T., VAN DER LINDEN, J. W. M., MELCHERS, W. J. G. & VERWEIJ, P. E. 2011. A new resistance mechanism emerging in clinical *Aspergillus fumigatus* isolates in The Netherlands *Mycoses,* 54**,** 56-57.

CARRASCO, L., MENDEZ , A. & JENSEN , H. E. 1996. Chronic bronchopulmonary aspergillosis in a horse with Cushing's syndrome. *Mycoses,* 39**,** 443-447.

CASSLE, S. E., LANDRAU-GIOVANNETTI, N., FARINA, L. L., LEONE, A., WELLEHAN, J. F. X., JR., STACY, N. I., THOMPSON, P., HERRING, H., MASE-GUTHRIE, B., BLAS-MACHADO, U., SALIKI, J. T., WALSH, M. T. & WALTZEK, T. B. 2016. Coinfection by Cetacean morbillivirus and *Aspergillus fumigatus* in a juvenile bottlenose dolphin (*Tursiops truncatus*) in the Gulf of Mexico. *Journal of Veterinary Diagnostic Investigation,* 28.

CHAMILOS, G., LUNA, M., LEWIS, R. E., BODEY, G. P., CHEMALY, R., TARRAND, J. J., SAFDAR, A., RAAD, I. I. & KONTOYIANNIS, D. P. 2006. Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989-2003. *Haematologica,* 91**,** 986-989.

CHEN, J., LI, H., LI, R., BU, D. & WAN, Z. 2005. Mutations in the cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *Journal of Antimicrobial Chemotherapy,* 55**,** 31-37.

CHONG, G. L., VAN DE SANDE, W. W., DINGEMANS, G. J., GAAJETAAN, G. R., VONK, A. G., HAYETTE, M. P., VAN TEGELEN, D. W., SIMONS, G. F. & RIJNDERS, B. J. 2015. Validation of a new *Aspergillus* real-time PCR assay for direct detection of *Aspergillus* and azole resistance of *Aspergillus fumigatus* on bronchoalveolar lavage fluid. *Journal of Clinical Microbiology,* 53**,** 868-74.

CHONG, G. M., VAN DER BEEK, M. T., VON DEM BORNE, P. A., BOELENS, J., STEEL, E., KAMPINGA, G. A., SPAN, L. F., LAGROU, K., MAERTENS, J. A., DINGEMANS, G. J., GAAJETAAN, G. R., VAN TEGELEN, D. W., CORNELISSEN, J. J., VONK, A. G. & RIJNDERS, B. J. 2016. PCR-based detection of *Aspergillus fumigatus Cyp51A* mutations on bronchoalveolar lavage: a multicentre validation of the AsperGenius assay(R) in 201 patients with haematological disease suspected for invasive aspergillosis. *Journal of Antimicrobial Chemotherapy,* 71**,** 3528-3535.

CHONG, G. M., VONK, A. G., MEIS, J. F., DINGEMANS, G. J., HOUBRAKEN, J., HAGEN, F., GAAJETAAN, G. R., VAN TEGELEN, D. W., SIMONS, G. F. & RIJNDERS, B. J. 2017. Interspecies discrimination of *A. fumigatus* and siblings *A. lentulus* and *A. felis* of the *Aspergillus* section *Fumigati* using the AsperGenius((R)) assay. *Diagnostic Microbiology and Infectious Disease,* 87**,** 247-252.

CHOUKRI, F., BOTTEREL, F., SITTERLE, E., BASSINET, L., FOULET, F., GUILLOT, J., COSTA, J. M., FAUCHET, N. & DANNAOUI, E. 2015. Prospective evaluation of azole resistance in *Aspergillus fumigatus* clinical isolates in France. *Medical Mycology,* 53**,** 593-6.

CHOWDHARY, A., KATHURIA, S., RANDHAWA, H. S., GAUR, S. N., KLAASSEN, C. H. & MEIS, J. F. 2012a. Isolation of multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR/L98H mutations in the cyp51A gene in India. *Journal of Antimicrobial Chemotherapy,* 67**,** 362-366.

CHOWDHARY, A., KATHURIA, S., XU, J. & MEIS, J. F. 2013. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health *PLoS Pathogens,* 9**,** e1003633.

CHOWDHARY, A., KATHURIA, S., XU, J., SHARMA, C., SUNDAR, G., SINGH, P. K., GAUR, S. N., HAGEN, F., KLAASSEN, C. H. & MEIS, J. F. 2012b. Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in the *cyp51A* gene in India. *PLoS One,* 7**,** e52871.

CHOWDHARY, A., SHARMA, C., KATHURIA, S., HAGEN, F. & MEIS, J. F. 2014a. Azole-resistant *Aspergillus fumigatus* with the environmental TR46/Y121F/T289A mutation in India. *Journal of Antimicrobial Chemotherapy,* 69**,** 555-7.

CHOWDHARY, A., SHARMA, C., KATHURIA, S., HAGEN, F. & MEIS, J. F. 2015. Prevalence and mechanism of triazole resistance in *Aspergillus fumigatus* in a referral chest hospital in Delhi, India and an update of the situation in Asia. *Frontiers in Microbiology,* 6**,** 428.

CHOWDHARY, A., SHARMA, C. & MEIS, J. F. 2017. Azole-resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. *The Journal of Infectious Diseases,* 216**,** S436-S444.

CHOWDHARY, A., SHARMA, C., VAN DEN BOOM, M., YNTEMA, J. B., HAGEN, F., VERWEIJ, P. E. & MEIS, J. F. 2014b. Multi-azole-resistant *Aspergillus fumigatus* in the environment in Tanzania. *Journal of Antimicrobial Chemotherapy,* 69**,** 2979-83.

CHRDLE, A., MUSTAKIM, S., BRIGHT-THOMAS, R. J., BAXTER, C. G., FELTON, T. & DENNING, D. W. 2012. *Aspergillus* bronchitis without significant immunocompromise. *Annals of the New York Academy of Sciences,* 1272**,** 73-85.

CHRYSSANTHOU, E. 1997. *In vitro* susceptibility of respiratory isolates of *Aspergillus* species to itraconazole and amphotericin B. Acquired resistance to Itraconazole. *Scandinavian Journal of Infectious Diseases,* 29**,** 509-512.

CHURCH, S., WYN-JONES, G., PARKS, A. H. & RITCHIE, H. E. 1986. Treatment of guttural pouch mycosis. *Equine Veterinary Journal,* 18**,** 362-365.

CHUTE, H. L., WITTER, J. F., ROUNTREE, J. L. & O'MEARA, D. C. 1955. The pathology of a fungous infection associated with a caponizing injury. *Journal of the American Veterinary Medical Association,* 127**,** 207-209.

CLAEYS, S., LEFEBVRE, J. B., SCHULLER, S., HAMAIDE, A. & CLERCX, C. 2006. Surgical treatment of canine nasal aspergillosis by rhinotomy combined with enilconazole infusion and oral itraconazole. *The Journal of Small Animal Practice,* 47**,** 320-4.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) 2018. M59 Epidemiology cutoff values of antifungal susceptibility testing Clinical and Laboratory Standards Institute: 2nd ed. CLSI supplement M59, Wayne, PA.

CLINICAL LABOARTORY STANDARDS INSTITUTE (CLSI) 2017. M38, 3rd edition *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi.* Clinical and Laboratory Standards Institute: CLSI, Wayne, PA.

COELHO, D., SILVA, S., VALE-SILVA, L., GOMES, H., PINTO, E., SARMENTO, A. & PINHEIRO, M. D. 2011. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. *Medical Mycology,* 49**,** 755-759.

COOK, W. 1968. The clinical features of guttural pouch mycosis in the horse. *Veterinary Record,* 83**,** 336-345.

CORK, S. C., ALLEY, M. R., JOHNSTONE, A. C. & STOCKDALE, P. H. 1999. Aspergillosis and other causes of mortality in the stitchbird in New Zealand. *Journal of Wildlife Diseases,* 35**,** 481-6.

CORTES, P. L., SHIVAPRASAD, H. L., KIUPEL, M. & SENTÍES-CUÉ, G. 2005. Omphalitis associated with *Aspergillus fumigatus* in poults. *Avian Diseases,* 49**,** 304-308.

COYNER, K. 2010. Otomycosis due to *Aspergillus* spp. in a dog: case report and literature review. *Veterinary Dermatology,* 21**,** 613-618.

CRAY, C., REAVILL, D., ROMAGNANO, A., VAN SANT, F., CHAMPAGNE, D., STEVENSON, R., ROLFE, V., GRIFFIN, C. & CLUBB, S. 2009a. Galactomannan assay and plasma protein electrophoresis findings in psittacine birds with aspergillosis. *Journal of Avian Medicine and Surgery,* 23**,** 125-35.

CRAY, C., WATSON, T., RODRIGUEZ, M. & ARHEART, K. L. 2009b. Application of galactomannan analysis and protein electrophoresis in the diagnosis of aspergillosis in avian species. *Journal of Zoo and Wildlife Medicine,* 40**,** 64-70.

DAGLEISH, M. P., PATTERSON, I. A. P., FOSTER, G., REID, R. J., LINTON, C. & BUXTON, D. 2006. Intracranial granuloma caused by asporogenic *Aspergillus fumigatus* in a harbour porpoise (*Phocoena phocoena*). *The Veterinary Record,* 159**,** 458-460.

DAGLEISH, M. R., FOSTER, G., HOWIE, F. E. & BARLEY, J. 2008. Fatal mycotic encephalitis caused by *Aspergillus fumigatus* in a northern bottlenose whale (*Hyperoodon ampullatus*). *The Veterinary Record,* 163**,** 602-604.

DALTON, J. R. & AINSWORTH, H. 2011. Mycotic keratoconjunctivitis in 12-day-old red-legged partridges (*Alectoris rufa*). *The Veterinary Record,* 168**,** 590.

DAY, M. J. 2009. Canine sino-nasal aspergillosis: parallels with human disease. *Medical Mycology,* 47**,** S315-S323.

DE FONTBRUNE, F. S., DENIS, B., MEUNIER, M., GARCIA-HERMOSO, D., BRETAGNE, S. & ALANIO, A. 2014. Iterative breakthrough invasive aspergillosis due to TR(34) /L98H azole-resistant *Aspergillus fumigatus* and *Emericella sublata* in a single hematopoietic stem cell transplant patient. *Transplant Infectious Disease,* 16**,** 687-91.

DE SOUTO, E. P. F., CARVALHO, G. S., FRADE, M. T. S., OLINDA, R. G., PESSOA, C. R. D. M., KOMMERS, G. D., SOUZA, A. P. & DANTAS, A. F. M. 2016. Bronchopulmonary aspergillosis associated with acute myocardial infarction in dog. *Acta Scientiae Veterinariae,* 44**,** 178-182.

DE VALK, H. A., MEIS, J. F., DE PAUW, B. E., DONNELLY, P. J. & KLAASSEN, C. H. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. *Journal of Clinical Microbiology,* 45**,** 1415-9.

DEEM, S. L. 2003. Fungal diseases of birds of prey. *Veterinary Clinics of North America: Exotic Animal Practice,* 6**,** 363-376.

DÉGI, J., RADBEA, G., BALABAN, S. & NICHITA, I. 2011. *Aspergillus* pneumonia in a Burmese cat: case study. *Lucrari Stiintifice - Universitatea de Stiinte Agricole a Banatului Timisoara, Medicina Veterinara,* 44**,** 278-281.

DELANEY, M. A., TERIO, K. A., COLEGROVE, K. M., BRIGGS, M. B. & KINSEL, M. J. 2013. Occlusive fungal tracheitis in 4 captive bottlenose dolphins (*Tursiops truncatus*). *Veterinary Pathology,* 50.

DELFS, K. C., HAWKINS, J. F. & HOGAN, D. F. 2009. Treatment of acute epistaxis secondary to guttural pouch mycosis with transarterial nitinol vascular occlusion plugs in three equids. *Journal of the American Veterinary Medical Association,* 235**,** 189-193.

DENNING, D. W. 1998. Invasive aspergillosis. *Clinical Infectious Diseases,* 26**,** 781-803; quiz 804-5.

DENNING, D. W. 2009. Chronic aspergillosis. *In:* LATGE, J. P. & STEINBACH, W. J. (eds.) *Aspergillus fumigatus and aspergillosis.* Washington: ASM Press.

DENNING, D. W., CADRANEL, J., BEIGELMAN-AUBRY, C., ADER, F., CHAKRABARTI, A., BLOT, S., ULLMANN, A. J., DIMOPOULOS, G., LANGE, C., EUROPEAN SOCIETY FOR CLINICAL, M., INFECTIOUS, D. & EUROPEAN RESPIRATORY, S. 2016. Chronic pulmonary aspergillosis: rationale and clinical guidelines for diagnosis and management. *European Respiratory Journal,* 47**,** 45-68.

DENNING, D. W., PARK, S., LASS-FLORL, C., FRACZEK, M. G., KIRWAN, M., GORE, R., SMITH, J., BUEID, A., MOORE, C. B., BOWYER, P. & PERLIN, D. S. 2011a. High-

frequency triazole resistance found In nonculturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. *Clinical Infectious Diseases,* 52**,** 1123-9.

DENNING, D. W., PLEUVRY, A. & COLE, D. C. 2011b. Global burden of chronic pulmonary aspergillosis as a sequel to pulmonary tuberculosis. *Bulletin of the World Health Organization,* 89**,** 864-872.

DENNING, D. W., VENKATESWARLU, K., OAKLEY, K. L., ANDERSON, M. J., MANNING, N. J., STEVENS, D. A., WARNOCK, D. W. & KELLY, S. L. 1997. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy,* 41**,** 1364-1368.

DES LIONS, J. A., GUILLOT, J., LEGRAND, E., BISSEAUD, O. & JENSEN, H. E. 2000. Aspergillosis involving the frontal sinus in a horse. *Equine Veterinary Education,* 12**,** 248-250.

DIXON, D. D. & WALSH, T. J. 1992. Human pathogenesis. *In:* BENNETT, J. W. & KLICH, M. A. (eds.) *Aspergillus, biology and industrial application.* Boston, Mass: Butterworth-Heinemann.

DOBESOVA, O., SCHWARZ, B., VELDE, K., JAHN, P., ZERT, Z. & BEZDEKOVA, B. 2012. Guttural pouch mycosis in horses: a retrospective study of 28 cases. *The Veterinary Record,* 171**,** 561.

DOMINGO, M., VISA, J., PUMAROLA, M., MARCO, A. J., FERRER, L., RABANAL, R. & KENNEDY, S. 1992. Pathologic and immunocytochemical studies of morbillivirus infection in striped dolphins (*Stenella coeruleoalba*). *Veterinary Pathology,* 29**,** 1-10.

DUNNE, K., HAGEN, F., POMEROY, N., MEIS, J. F. & ROGERS, T. R. 2017. Intercountry transfer of triazole-resistant *Aspergillus fumigatus* on plant bulbs. *Clinical Infectious Diseases,* 65**,** 147-149.

DYAR, P. M., FLETCHER, O. J. & PAGE, R. K. 1984. Aspergillosis in turkeys associated with use of contaminated litter. *Avian Diseases,* 28**,** 250-255.

ELLIS, D. 2017. *Mycology online* [Online]. Adelaide, SA: The University of Adelaide. Available: https://mycology.adelaide.edu.au/laboratory/ 2017].

ESCRIBANO, P., PELÁEZ, T., MUÑOZ, P., BOUZA, E. & GUINEA, J. 2013. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? *Antimicrobial Agents and Chemotherapy,* 57**,** 2815-2820.

ESCRIBANO, P., RECIO, S., PELAEZ, T., BOUZA, E. & GUINEA, J. 2011. *Aspergillus fumigatus* strains with mutations in the *cyp51A* gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. *Antimicrobial Agents and Chemotherapy,* 55**,** 2460-2.

ESPINEL-INGROFF, A., CHOWDHARY, A., GONZALEZ, G. M., LASS-FLORL, C., MARTIN-MAZUELOS, E., MEIS, J., PELAEZ, T., PFALLER, M. A. & TURNIDGE, J. 2013. Multicenter study of isavuconazole MIC distributions and epidemiological cutoff values for *Aspergillus* spp. for the CLSI M38-A2 broth microdilution method. *Antimicrobial Agents and Chemotherapy,* 57**,** 3823-8.

ESPINELL-INGROFF, A., DIEKEMA, D. J., FOTHERGILL, A., JOHNSON, E., PELAEZ, T., PFALLER, M. A., RINALDI, M. G., CANTON, E. & TURNIDGE, J. 2010. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Journal of Clinical Microbiology,* 48**,** 3251-3257.

EUCAST 2017. Antifungal agents breakpoint tables for interpretation of MICs. *Aspergillus spp.* Sweden: EUCAST.

FISCHER, J., VAN KONINGSBRUGGEN-RIETSCHEL, S., RIETSCHEL, E., VEHRESCHILD, M. J., WISPLINGHOFF, H., KRONKE, M. & HAMPRECHT, A. 2014. Prevalence and molecular characterization of azole resistance in *Aspergillus* spp. isolates from German cystic fibrosis patients. *Journal of Antimicrobial Chemotherapy,* 69**,** 1533-6.

FOX, J. G., MURPHY, J. C. & SHALEV, M. 1978. Systemic fungal infections in cats. *Journal of the American Veterinary Medical Association,* 173**,** 1191-1195.

FRACZEK, M. G., BROMLEY, M. & BOWYER, P. 2011. An improved model of the *Aspergillus fumigatus* CYP51A protein *Antimicrobial Agents and Chemotherapy,* 55**,** 2483-2486.

FRANCA, M., CRAY, C. & SHIVAPRASAD, H. L. 2012. Serologic testing for aspergillosis in commercial broiler chickens and turkeys. *Avian Diseases,* 56**,** 160-164.

FRISVAD, J. C. & LARSEN, T. O. 2016. Extrolites of *Aspergillus fumigatus* and other pathogenic species in *Aspergillus* section *Fumigati*. *Frontiers in Microbiology,* 6**,** 1-14.

FRYE, F. L. & DUTRA, F. R. 1974. Mycotic granulomata involving the forefeet of a turtle. *Veterinary Medicine, Small Animal Clinician,* 69**,** 1554-1556.

FURROW, E. & GROMAN, R. P. 2009. Intranasal infusion of clotrimazole for the treatment of nasal aspergillosis in two cats. *Journal of the American Veterinary Medical Association,* 235**,** 1188-1193.

GARCIA-EFFRON, G., DILGER, A., ALCAZAR-FUOLI, L., PARK, S., MELLADO, E. & PERLIN, D. S. 2008. Rapid detection of triazole antifungal resistance in *Aspergillus fumigatus*. *Journal of Clinical Microbiology,* 46**,** 1200-6.

GARCIA, M. E., DURAN, C., CRUZADO, M., ANDRINO, M. & BLANCO, J. L. 2004. Evaluation of molecular and immunological techniques for the diagnosis of mammary aspergillosis in ewes. *Veterinary Microbiology,* 98**,** 17-21.

GARCIA, R. S., WHEAT, L. J., COOK, A. K., KIRSCH, E. J. & SYKES, J. E. 2012. Sensitivity and specificity of a blood and urine galactomannan antigen assay for diagnosis of systemic aspergillosis in dogs. *Journal of Veterinary Internal Medicine,* 26**,** 911-9.

GIRLING, S. J. & FRASER, M. A. 2009. Treatment of *Aspergillus* species infection in reptiles with itraconazole at metabolically scaled doses. *The Veterinary Record,* 165**,** 52-54.

GISI, U. 2014. Assessment of selection and resistance risk for demethylation inhibitor fungicides in *Aspergillus fumigatus* in agriculture and medicine: a critical review. *Pest Management Science,* 70**,** 352-364.

GLASS, N. L. & DONALDSON, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology,* 61**,** 1323-1330.

GOODALL, S. A., LANE, J. G. & WARNOCK, D. W. 1984. The diagnosis and treatment of a case of nasal aspergillosis in a cat. *The Journal of Small Anim Practice,* 25**,** 627-633.

GREENBERGER, P. A. & PATTERSON, R. 1988. Allergic bronchopulmonary aspergillosis and the evaluation of the patient with asthma. *The Journal of Allergy and Clinical Immunology,* 81**,** 646-50.

GROCH, K. R., DIAZ-DELGADO, J., SACRISTAN, C., OLIVEIRA, D. E., SOUZA, G., SANCHEZ-SARMIENTO, A. M., COSTA-SILVA, S., MARIGO, J., CASTILHO, P. V., CREMER, M. J., RODRIGUES HOFFMANN, A., ESPERON, F. & CATAO-DIAS, J. L. 2018. Pulmonary and systemic fungal infections in an Atlantic spotted dolphin and a Bryde's whale, Brazil. *Diseases of Aquatic Organisms,* 128**,** 73-79.

GUARRO, J., KALLAS, E. G., GODOY, P., KARENINA, A., GENÉ, J., STCHIGEL, A. & COLOMBO, A. L. 2002. Cerebral aspergillosis caused by *Neosartorya hiratsukae*, Brazil. *Emerging Infectious Diseases,* 8**,** 989-991.

GUEGAN, H., CHEVRIER, S., BELLEGUIC, C., DENEUVILLE, E., ROBERT-GANGNEUX, F. & GANGNEUX, J.-P. 2018. Performance of molecular approaches for *Aspergillus* detection and azole resistance surveillance in cystic fibrosis. *Frontiers in Microbiology,* 9**,** 531.

GUITARD, J., SENDID, B., THOREZ, S., GITS, M. & HENNEQUIN, C. 2012. Evaluation of a recombinant antigen-based enzyme immunoassay for the diagnosis of noninvasive aspergillosis. *Journal of Clinical Microbiology,* 50**,** 762-5.

GYOTOKU, H., IZUMIKAWA, K., IKEDA, H., TAKAZONO, T., MORINAGA, Y., NAKAMURA, S., IMAMURA, Y., NISHINO, T., MIYAZAKI, T., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., YASUOKA, A., YAGUCHI, T., OHNO, H., MIYZAKI, Y., KAMEI, K., KANDA, T. & KOHNO, S. 2012. A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. *Med Mycol.*, 50**,** 631-636.

HAGIWARA, D., TAKAHASHI, H., FUJIMOTO, M., SUGAHARA, M., MISAWA, Y., GONOI, T., ITOYAMA, S., WATANABE, A. & KAMEI, K. 2016. Multi-azole resistant *Aspergillus fumigatus* harboring *Cyp51A* TR46/Y121F/T289A isolated in Japan. *J Infect Chemother*, 22**,** 577-9.

HAINES, J. 1995. *Aspergillus* in compost: straw man or fatal flaw? *Biocycle*, 6**,** 32-35.

HAMILTON, H. L., WHITLEY, R. D. & MCLAUGHLIN, S. A. 2000. Exophthalmos secondary to aspergillosis in a cat. *Journal of the American Animal Hospital Association*, 36**,** 343-347.

HAMPRECHT, A., BUCHHEIDT, D., VEHRESCHILD, J. J., CORNELY, O. A., SPIESS, B., PLUM, G., HALBSGUTH, T. V., KUTSCH, N., STIPPEL, D., KAHL, P., PERSIGEHL, T., STEINBACH, A., BOS, B., HALLEK, M. & VEHRESCHILD, M. J. 2012. Azole-resistant invasive aspergillosis in a patient with acute myeloid leukaemia in Germany. *Euro Surveillance*, 17**,** 20262.

HARGROVE, T. Y., WAWRZAK, Z., LAMB, D. C., GUENGERICH, F. P. & LEPESHEVA, G. I. 2015. Structure-functional characterization of cytochrome P450 sterol 14α-demethylase (*CYP51B*) from *Aspergillus fumigatus* and molecular basis for the development of antifungal drugs. *The Journal of Biological Chemistry* 290**,** 23916-23934.

HARVEY, C. 1984. Nasal aspergillosis and penicillosis in dogs: results of treatment with thiabendazole. *Journal of the American Veterinary Medical Association*, 184.

HATTEL, A. L., DRAKE, T. R., ANDERHOLM, B. J. & MCALLISTER, E. S. 1991. Pulmonary aspergillosis associated with acute enteritis in a horse. *Journal of the American Veterinary Medical Association,* 199**,** 589-590.

HAZELL, K. L., SWIFT, I. M. & SULLIVAN, N. 2011. Successful treatment of pulmonary aspergillosis in a cat. *Australian Veterinary Journal,* 89**,** 101-104.

HODIAMONT, C. J., DOLMAN, K. M., TEN BERGE, I. J., MELCHERS, W. J., VERWEIJ, P. E. & PAJKRT, D. 2009. Multiple-azole-resistant *Aspergillus fumigatus* osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. *Medical Mycology,* 47**,** 217-20.

HOLLOMON, D. 2017. Does agricultural use of azole fungicides contribute to resistance in the human pathogen *Aspergillus fumigatus*? *Pest Management Science,* 73**,** 1987-1993.

HONG, S. B., GO, S. J., SHIN, H. D., FRISVAD, J. C. & SAMSON, R. A. 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia,* 97**,** 1316-1329.

HONG, S. B., KIM, D. H., PARK, I. C., CHOI, Y. J., SHIN, H. D. & SAMSON, R. 2010. Re-identification of *Aspergillus fumigatus* sensu lato based on a new concept of species delimitation. *The Journal of Microbiology,* 48**,** 607-15.

HOPE, W. W., WALSH, T. J. & DENNING, D. W. 2005. The invasive and saprophytic syndromes due to *Aspergillus* spp. *Medical Mycology,* 43**,** S207-S238.

HOPPES, S., GURFIELD, N., FLAMMER, K., COLITZ, C. & FISHER, P. 2000. Mycotic keratitis in a blue-fronted Amazon parrot (*Amazona aestiva*). *Journal of Avian Medicine and Surgery,* 14**,** 185-189.

HOWARD, S. J., CERAR, D., ANDERSON, M. J., ALBARRAG, M. C., PERLIN, D. S. & DENNING, D. W. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerging Infectious Diseases,* 15**,** 1068-1076.

HOWARD, S. J., PASQUALOTTO, A. C., ANDERSON, M. J., LEATHERBARROW, H., ALBARRAG, A. M., HARRISON, E., GREGSON, L., BOWYER, P. & DENNING, D. W. 2013. Major variations in *Aspergillus fumigatus* arising within aspergillomas in chronic pulmonary aspergillosis. *Mycoses,* 56**,** 434-41.

HOWARD, S. J., WEBSTER, I., MOORE, C. B., GARDINER, R. E., PARK, S., PERLIN, D. S. & DENNING, D. W. 2006. Multi-azole resistance in *Aspergillus fumigatus*. *International Journal of Antimicrobial Agents,* 28**,** 450-3.

HUBKA, V., BARRS, V., DUDOVA, Z., SKLENAR, F., KUBATOVA, A., MATSUZAWA, T., YAGUCHI, T., YOSHIKAZU, H., NOVAKOVA, A., FRISVAD, J. C., TALBOT, J. J. & KOLARIK, M. 2018. Unravelling species boundaries in the *Aspergillus virdinutans* complex (section *Fumigati*): opportunistic human and anmal pathogens capable of interspecific hybridization. *Persoonia,* 41, in press, accepted for publication 14th March 2018.

HUNTER, B. & NATION, P. N. 2011. Mycotic encephalitis, sinus osteomyelitis, and guttural pouch mycosis in a 3-year-old Arabian colt. *The Canadian Veterinary Journal,* 52**,** 1339-1341.

HURST, S. F., BERKOW, E. L., STEVENSON, K. L., LITVINTSEVA, A. P. & LOCKHART, S. R. 2017. Isolation of azole-resistant *Aspergillus fumigatus* from the environment in the south-eastern USA. *Journal of Antimicrobial Chemotherapy,* 72**,** 2443-2446.

IDELEVICH, E. A., GROSS, U., BECKER, K. & BADER, O. 2018. Comparative evaluation of different gradient diffusion tests for detection of azole resistance in *Aspergillus fumigatus*. *Diagnostic Microbiology and Infectious Disease*.

ITO, K., YAMAYA, Y., ASANO, K., SANO, T., WATARI, T., SIBUYA, H., SATO, T. & TANAKA, S. 2011. A case of pulmonary aspergilloma in a Border Collie. *Japanese Journal of Veterinary Anesthesia and Surgery,* 42**,** 13-16.

JACOBSON, E. R., CHEATWOOD, J. L. & MAXWELL, L. K. 2000. Mycotic diseases of reptiles. *Seminars in Avian and Exotic Pet Medicine,* 9**,** 94-101.

JEANVOINE, A., ROCCHI, S., REBOUX, G., CRINI, N., CRINI, G. & MILLON, L. 2017. Azole-resistant *Aspergillus fumigatus* in sawmills of Eastern France. *J Appl Microbiol,* 123**,** 172-184.

JENKINS, J. Aspergillosis. Annual Conference of the Association of Avian Veterinarians, 1991 Chicago, IL. 328.

JENSEN, H. E., OLSEN, S. N. & AALBAEK, B. 1994. Gastrointestinal aspergillosis and zygomycosis of cattle. *Veterinary Pathology,* 31**,** 28-36.

JENSEN, H. E., SCHONHEYDER, H. & JORGENSEN, J. B. 1990. Intestinal and pulmonary mycotic lymphadenitis in cattle. *Journal of Comparative Pathology,* 102**,** 345-355.

JOHNSON, L., DRAZENOVICH, T., HERRERA, M. & WISNER, E. 2006. Results of rhinoscopy alone or in conjunction with sinuscopy in dogs with aspergillosis: 46 cases (2001–2004). *Journal of the American Veterinary Medical Association,* 228**,** 738-742.

JONES, M. P. & OROSZ, S. E. 2000. The diagnosis of aspergillosis in birds. *Seminars in Avian and Exotic Pet Medicine,* 9**,** 52-58.

JOSEPH, B. E., CORNELL, L. H., SIMPSON, J. G., MIGAKI, G. & GRINER, L. 1986. Pulmonary aspergillosis in three species of dolphin. *Zoo Biology,* 5.

JURCZYNSKI, K., GRUBER-DUJARDIN, E., WIDMER, D., KAUP, F. J. & MÄTZ-RENSING, K. 2012. Invasive aspergillosis in a putty-nosed monkey (*Cercopithecus nictitans*) with adrenocortical Cushing's syndrome. *Journal of Medical Primatology,* 41**,** 172-175.

KANO, R., ITAMOTO, K., OKUDA, M., INOKUMA, H., HASEGAWA, A. & BALAJEE, S. A. 2008. Isolation of *Aspergillus udagawae* from a fatal case of feline orbital aspergillosis. *Mycoses,* 51**,** 360-361.

KANO, R., KOHATA, E., TATEISHI, A., MURAYAMA, S. Y., HIROSE, D., SHIBATA, Y., KOSUGE, Y., INOUE, H., KAMATA, H. & HASEGAWA, A. 2015. Does farm fungicide use induce azole resistance in *Aspergillus fumigatus*? *Med Mycol,* 53**,** 174-7.

KANO, R., SHIBAHASHI, A., FUJINO, Y., SAKAI, H., MORI, T., TSUJIMOTO, H., YANAI, T. & HASEGAWA, A. 2013. Two cases of feline orbital aspergillosis due to *Aspergillus udagawae* and *A. viridinutans*. *The Journal of Veterinary Medical Science,* 75**,** 7-10.

KAWAZU, M., KANDA, Y., NANNYA, Y., AOKI, K., KUROKAWA, M., CHIBA, S., MOTOKURA, T., HIRAI, H. & OGAWA, S. 2004. Prospective comparison of the diagnostic potential of real-time PCR, double-sandwich enzyme-linked immunosorbent assay for galactomannan, and a (1→3)-β-d-glucan test in weekly screening for invasive aspergillosis in patients with hematological disorders *Journal of Clinical Microbiology,* 42**,** 2733-2741.

KENDALL, A., BRÖJER, J., KARLSTAM, E. & PRINGLE, J. 2008. Enilconazole treatment of horses with superficial *Aspergillus* spp. rhinitis. *Journal of veterinary internal medicine* 22**,** 1239-1242.

KHARE, R., GUPTA, S., ARIF, S., JENTOFT, M. E., DEZIEL, P. J., RODEN, A. C., WILHELM, M. P., RAZONABLE, R. R. & WENGENACK, N. L. 2014. Misidentification of *Neosartorya pseudofischeri* as *Aspergillus fumigatus* in a lung transplant patient. *Journal of Clinical Microbiology,* 52**,** 2722-2725.

KIDD, S. E., GOEMAN, E., MEIS, J. F., SLAVIN, M. A. & VERWEIJ, P. E. 2015. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses,* 58**,** 350-355.

KIDD, S. E., WALKER, D., HAREMZA, E. & MORRIS, A. 2017. *Mycology QAP update* [Online]. The Royal College of Pathologies of Australia. Available: https://www.rcpa.edu.au/getattachment/25620d8b-928f-4552-86db-d5bcaeaad23b/Sarah-Kidd_Mycology-QAP-Update-Antifungal-Suscepti.aspx.

KIM, S. H., YONG, H. C., YOON, J. H., YOUN, H. Y., YOSHIOKA, N., KANO, R. & HASEGAWA, A. 2003. *Aspergillus niger* pulmonary infection in a dog. *The Journal of Veterinary Medical Science,* 65**,** 1139-1140.

KIRKPATRICK, R. M. 1982. Mycotic cystitis in a male cat. *Veterinary Medicine, Small Animal Clinician,* 77**,** 1365-1371.

KLAASSEN, C. H., DE VALK, H. A., CURFS-BREUKER, I. M. & MEIS, J. F. 2010. Novel mixed-format real-time PCR assay to detect mutations conferring resistance to triazoles in *Aspergillus fumigatus* and prevalence of multi-triazole resistance among clinical isolates in the Netherlands. *Journal of Antimicrobial Chemotherapy,* 65**,** 901-5.

KNUDTSON, W. U. & KIRKBRIDE, C. A. 1992. Fungi associated with bovine abortion in the northern plains states (USA). *Journal of Veterinary Diagnostic Investigation,* 4**,** 181-185.

KÖHLER, H., KUTTIN, E., KAPLAN, W., BURTSCHER, H., GRÜNBERG, W. & SWOBODA, R. 1978. Occurence of systemic mycoses in animals in Austria. *Zentralbl Veterinarmed B,* 25**,** 785-799.

KOLWIJCK, E., VAN DER HOEVEN, H., DE SÉVAUX, R. G. L., TEN OEVER, J., RIJSTENBERG, L. L., VAN DER LEE, H. A., ZOLL, J., MELCHERS, W. J. & VERWEIJ, P. E. 2016. Voriconazole-susceptible and voriconazole-resistant

Aspergillus fumigatus coinfection. *American Journal of Respiratory and Critical Care Medicine,* 193**,** 927-929.

KONTOYIANNIS, D. P., MARR, K. A., PARK, B. J., ALEXANDER, B. D., ANAISSIE, E. J., WALSH, T. J., ITO, J., ANDES, D. R., BADDLEY, J. W., BROWN, J. M., BRUMBLE, L. M., FREIFELD, A. G., HADLEY, S., HERWALDT, L. A., KAUFFMAN, C. A., KNAPP, K., LYON, G. M., MORRISON, V. A., PAPANICOLAOU, G., PATTERSON, T. F., PERL, T. M., SCHUSTER, M. G., WALKER, R., WANNEMUEHLER, K. A., WINGARD, J. R., CHILLER, T. M. & PAPPAS, P. G. 2010. Prospective surveillance for invasive fungal infections in hematopoeitic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSET) Database. *Clinical Infectious Diseases,* 50**,** 1091-1100.

KOO, S., BRYAR, J. M., PAGE, J. H., BADEN, L. R. & MARTY, F. M. 2009. Diagnostic performance of the (1¡3)-beta-D-glucan assay for invasive fungal disease. *Clinical Infectious Diseases,* 49.

KUCK, K. H. & RUSSELL, P. E. FRAC: combined resistance risk assessment. *In:* BRYSON, R. J., BURNETT, F. J., FOSTER, V., FRAAIJE, B. A. & KENNEDY, R., eds. Proceedings of the Association of Applied Biologists Meeting, "Fungicide Resistance: Are we Winning the Battle but Losing the War?", 7-8 September 2006 Edinburgh, UK. 3-10.

KURUP, V. P. & KUMAR, A. 1991. Immunodiagnosis of aspergillosis. *Clinical Microbiology Reviews,* 4**,** 439-56.

KUSUYA, Y., TAKAHASHI-NAKAGUCHI, A., TAKAHASHI, H. & YAGUCHI, T. 2015. Draft genome sequence of the pathogenic filamentous fungus *Aspergillus udagawae* strain IFM 46973T. *Genome Announcements,* 3**,** e00834-15.

LABELLE, A. L., HAMOR, R. E., BARGER, A. M., MADDOX, C. W. & BREAUX, C. B. 2009. *Aspergillus flavus* keratomycosis in a cat treated with topical 1% voriconazole solution. *Veterinary Opthalmology,* 12**,** 48-52.

LAGO, M., AGUIAR, A., NATARIO, A., FERNANDES, C., FARIA, M. & PINTO, E. 2014. Does fungicide application in vineyards induce resistance to medical azoles in *Aspergillus* species? *Environmental Monitoring and Assessment,* 186**,** 5581-93.

LAMOTH, F. 2016. *Aspergillus fumigatus*-related species in clinical practice. *Front Microbiol,* 7**,** 683.

LAMOTH, F. & ALEXANDER, B. D. 2015. Comparing Etest and broth microdilution for antifungal susceptibility testing of the most-relevant pathogenic molds. *Journal of Clinical Microbiology,* 53**,** 3176-81.

LANE, J. G. 1989. The management of guttural pouch mycosis. *Equine Veterinary Journal,* 21**,** 321-324.

LAS HERAS, A., DOMÍNGUEZ, L., LÓPEZ, I., PAYÁ, M. J., PEÑA, L., MAZZUCCHELLI, F., GARCÍA, L. A. & FERNÁNDEZ-GARAYZÁBAL, J. F. 2000. Intramammary *Aspergillus fumigatus* infection in dairy ewes associated with antibiotic dry therapy. *Vet Rec,* 147**,** 578-580.

LATGÉ, J.-P. 1999. *Aspergillus fumigatus* and Aspergillosis. *Clinical Microbiology Reviews,* 12**,** 310-350.

LATGÉ, J. P., PARIS, S., SARFATI, J., DEBEAUPUIS, J. P., BEAUVAIS, A., JATON-OGAY, K. & MONOD, M. 1997. Infectivity of *Aspergillus fumigatus*. *In:* H., V. B., STEVENS, D. A. & ODDS, F. C. (eds.) *Host-fungus interplay.* . Bethesda, Md: National Foundation for Infectious Diseases.

LAUFER, P., FINK, J. N., BRUNS, W. T., UNGER, G. F., KALBFLEISCH, J. H., GREENBERGER, P. A. & PATTERSON, R. 1984. Allergic bronchopulmonary aspergillosis in cystic fibrosis. *The Journal of Allergy and Clinical Immunology,* 73**,** 44-8.

LAVERGNE, R. A., CHOUAKI, T., HAGEN, F., TOUBLANC, B., DUPONT, H., JOUNIEAUX, V., MEIS, J. F., MORIO, F. & LE PAPE, P. 2017. Home environment as a source of life-threatening azole-resistant *Aspergillus fumigatus* in immunocompromised patients. *Clin Infect Dis,* 64**,** 76-78.

LAVERGNE, R. A., MORIO, F., FAVENNEC, L., DOMINIQUE, S., MEIS, J. F., GARGALA, G., VERWEIJ, P. E. & LE PAPE, P. 2015. First description of azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation in France. *Antimicrobial Agents and Chemotherapy,* 59**,** 4331-5.

LE PAPE, P., LAVERGNE, R. A., MORIO, F. & ALVAREZ-MORENO, C. 2016. Multiple fungicide-driven alterations in azole-resistant

Aspergillus fumigatus, Colombia, 2015. *Emerg Infect Dis,* 22**,** 156-157.

LEONARDELLI, F., THEILL, L., NARDIN, M. E., MACEDO, D., DUDIUK, C., MENDEZ, E., GAMARRA, S. & GARCIA-EFFRON, G. 2017. First itraconazole resistant *Aspergillus fumigatus* clinical isolate harbouring a G54E substitution in *Cyp51Ap* in South America. *Rev Iberoam Micol,* 34**,** 46-48.

LEPAGE, O. M. & PICCOT-CREZOLLET, C. 2005. Transarterial coil embolisation in 31 horses (1999–2002) with guttural pouch mycosis: a 2-year follow-up. *Equine Veterinary Journal*, 37**,** 430-434.

LESTRADE, P. P., MEIS, J. F., ARENDS, J. P., VAN DER BEEK, M. T., DE BRAUWER, E., VAN DIJK, K., DE GREEFF, S. C., HAAS, P. J., HODIAMONT, C. J., KUIJPER, E. J., LEENSTRA, T., MULLER, A. E., OUDE LASHOF, A. M., RIJNDERS, B. J., ROELOFSEN, E., ROZEMEIJER, W., TERSMETTE, M., TERVEER, E. M., VERDUIN, C. M., WOLFHAGEN, M. J., MELCHERS, W. J. & VERWEIJ, P. E. 2016. Diagnosis and management of aspergillosis in the Netherlands: a national survey. *Mycoses*, 59**,** 101-7.

LESTRADE, P. P., VAN DER VELDEN, W., BOUWMAN, F., STOOP, F. J., BLIJLEVENS, N. M. A., MELCHERS, W. J. G., VERWEIJ, P. E. & DONNELLY, J. P. 2018. Epidemiology of invasive aspergillosis and triazole-resistant *Aspergillus fumigatus* in patients with haematological malignancies: a single-centre retrospective cohort study. *Journal of Antimicrobial Chemotherapy*.

LÉVEILLÉ, R., HARDY, J., ROBERTSON, J. T., WILLIS, A. M., BEARD, W. L., WEISBRODE, S. E. & LEPAGE, O. M. 2000. Transarterial coil embolization of the internal and external carotid and maxillary arteries for prevention of hemorrhage from guttural pouch mycosis in horses. *Veterinary Surgery*, 29**,** 389-397.

LEWIS, D. F., WISEMAN, A. & TARBIT, M. H. 1999. Molecular modelling of lanosterol 14 alpha-demethylase (*CYP51*) from *Saccharomyces cerevisiae* via homology with *CYP102*, a unique bacterial cytochrome P450 isoform: quantitative structure-activity relationships (QSARs) within two related series of antifungal azole derivatives. *Journal of Enzyme Inhibition*, 14**,** 175–192.

LIU, M., ZENG, R., ZHANG, L., LI, D., LV, G., SHEN, Y., ZHENG, H., ZHANG, Q., ZHAO, J., ZHENG, N. & LIU, W. 2015. Multiple *cyp51A*-based mechanisms identified in azole-resistant isolates of *Aspergillus fumigatus* from China. *Antimicrobial Agents and Chemotherapy*, 59**,** 4321-5.

LIU, M., ZHENG, N., LI, D., ZHENG, H., ZHANG, L., GE, H. & LIU, W. 2016. cyp51A-based mechanism of azole resistance in *Aspergillus fumigatus*: Illustration by a new 3D Structural Model of *Aspergillus fumigatus CYP51A* protein. *Medical Mycology,* 54**,** 400-408.

LOCKHART, S. R., FRADE, J. P., ETIENNE, K. A., PFALLER, M. A., DIEKEMA, D. J. & BALAJEE, S. A. 2011. Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the *cyp51A* gene. *Antimicrobial Agents and Chemotherapy,* 55**,** 4465-8.

LYSKOVA, P., HUBKA, V., SVOBODOVA, L., BARRS, V., DHAND, N. K., YAGUCHI, T., MATSUZAWA, T., HORIE, Y., KOLARIK, M., DOBIAS, R. & HAMAL, P. 2018. Antifungal susceptibility of the *Aspergillus viridinutans* complex: comparison of two *in vitro* methods. *Antimicrobial Agents and Chemotherapy,* 62.

MAERTENS, J. A., RAAD, I. I., MARR, K. A., PATTERSON, T. F., KONTOYIANNIS, D. P., CORNELY, O. A., BOW, E. J., RAHAV, G., NEOFYTOS, D., AOUN, M., BADDLEY, J. W., GILADI, M., HEINZ, W. J., HERBRECHT, R., HOPE, W., KARTHAUS, M., LEE, D.-G., LORTHOLARY, O., MORRISON, V. A., OREN, I., SELLESLAG, D., SHOHAM, S., THOMPSON, G. R., LEE, M., MAHER, R. M., SCHMITT-HOFFMANN, A.-H., ZEIHER, B. & ULLMANN, A. J. 2016. Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by Aspergillus and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial. *The Lancet,* 387**,** 760-769.

MALEJCZYK, K., SIGLER, L., GIBAS, C. F. & SMITH, S. W. 2013. Invasive sino-orbital mycosis in an aplastic anemia patient caused by *Neosartorya laciniosa*. *Journal of Clinical Microbiology,* 51**,** 1316-9.

MANN, P. A., PARMEGIANI, R. M., WEI, S. Q., MENDRICK, C. A., LI, X., LOEBENBERG, D., DIDOMENICO, B., HARE, R. S., WALKER, S. S. & MCNICHOLAS, P. M. 2003. Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14 -demethylase. *Antimicrobial Agents and Chemotherapy,* 47**,** 577-581.

MARR, K. A., LAVERDIERE, M., GUGEL, A. & LEISENRING, W. 2005. Antifungal therapy decreases sensitivity of the *Aspergillus* galactomannan enzyme immunoassay. *Clinical Infectious Diseases,* 40**,** 1762-1769.

MARTIN, M. P., BOUCK, K. P., HELM, J., DYKSTRA, M. J., WAGES, D. P. & J., B. H. 2007. Disseminated *Aspergillus flavus i*nfection in broiler breeder pullets. *Avian Diseases,* 51**,** 626-631.

MATHEWS, K. & SHARP, N. 2006. Aspergillosis and penicilliosis. *In:* GREENE, C. (ed.) *Infectious diseases of the dog and cat.* St. Louise, MO: Saunders Elsevier.

MCCAUSLAND, I. P. 1972. Systemic mycoses of two cats. *New Zealand Veterinary Journal,* 20**,** 10-12.

MCCLENNY, N. 2005. Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: the traditional approach. *Medical Mycology,* 43**,** 125-128.

MEERSSEMAN, W., VANDECASTEELE, S. J., WILMER, A., VERBEKEN, E., PEETERMANS, W. E. & VAN WIJNGAERDEN, E. 2004. Invasive aspergillosis in critically ill patients without malignancy. *American Journal of Respiratory and Critical Care Medicine,* 170**,** 621-5.

MEIS, J. F., CHOWDHARY, A., RHODES, J. L., FISHER, M. C. & VERWEIJ, P. E. 2016. Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus*. *Philos Trans R Soc Lond B Biol Sci,* 371.

MELER, E., DUNN, M. & LECUYER, M. 2008. A retrospective study of canine persistent nasal disease: 80 cases (1998-2003). *The Canadian Veterinary Journal,* 49.

MELETIADIS, J., MOUTON, J. W., MEIS, J. F. G. M., BOUMAN, B. A. & VERWEIJ, P. E. 2002. Comparison of the Etest and the Sensititre colorimetric methods with the NCCLS proposed standard for antifungal susceptibility testing of *Aspergillus* species. *Journal of Clinical Microbiology,* 40**,** 2876-2885.

MELLADO, E., ALCAZAR-FUOLI, L., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2011. Role of *Aspergillus lentulus* 14-alpha sterol demethylase (*Cyp51A*) in azole drug susceptibility. *Antimicrobial Agents and Chemotherapy,* 55**,** 5459-68.

MELLADO, E., DE LA CAMARA, R., BUENDÍA, B., RODRIGUEZ-TUDELA, J. L. & CUENCA-ESTRELLA, M. 2013. Breakthrough pulmonary *Aspergillus fumigatus* infection with multiple triazole resistance in a Spanish patient with chronic myeloid leukemia. *Revista Iberoamericana de Micología,* 30**,** 64-68.

MELLADO, E., DIAZ-GUERRA, T. M., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2001. Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *Journal of Clinical Microbiology,* 39**,** 2431-2438.

MELLADO, E., GARCIA-EFFRON, G., ALCAZAR-FUOLI, L., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2004. Substitutions at Methionine 220 in the 14α-Sterol Demethylase (*Cyp51A*) of *Aspergillus fumigatus* Are Responsible for Resistance In Vitro to Azole Antifungal Drugs. *Antimicrobial Agents and Chemotherapy,* 48**,** 2747-2750.

MELLO, E., POSTERARO, B., VELLA, A., DE CAROLIS, E., TORELLI, R., D'INZEO, T., VERWEIJ, P. E. & SANGUINETTI, M. 2017. Susceptibility testing of common and uncommon *Aspergillus* Species against posaconazole and other mold-active antifungal azoles using the Sensititre method. *Antimicrob Agents Chemother,* 61.

MENNINK-KERSTEN, M. A., DONNELLY, J. P. & VERWEIJ, P. E. 2004. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. *The Lancet Infectious Diseases,* 4**,** 349-357.

MIGAKI, G. 1983. Mycotic diseases in marine mammals. *In:* HOWARD, E. B. (ed.) *Pathobiology of marine mammal diseases.* Boca Rotan, FL: CRC Press.

MILLER, D. L., RADI, Z. A., STIVER, S. L. & THORNHILL, T. D. 2004. Cutaneous and pulmonary mycosis in green anacondas (*Euncectes murinus*). *Journal of Zoo and Wildlife Medicine,* 35**,** 557-561.

MOHAMMADI, F., HASHEMI, S. J., ZOLL, J., MELCHERS, W. J., RAFATI, H., DEHGHAN, P., REZAIE, S., TOLOOE, A., TAMADON, Y., VAN DER LEE, H. A., VERWEIJ, P. E. & SEYEDMOUSAVI, S. 2016. Quantitative analysis of single-nucleotide polymorphism for rapid detection of TR34/L98H- and TR46/Y121F/T289A-positive *Aspergillus fumigatus* isolates obtained from patients in Iran from 2010 to 2014. *Antimicrob Agents Chemother,* 60**,** 387-92.

MOHAMMED, M. A., SOKKAR, S. M., BATRAWI, A., REFAIE, M. K. & AHMED, A. A. 1980. Natural and experimental nodular dermatomycosis in chickens. *Avian Pathology,* 9**,** 185-92.

MONTESINOS, I., ARGUDIN, M. A., HITES, M., AHAJJAM, F., DODEMONT, M., DAGYARAN, C., BAKKALI, M., ETIENNE, I., JACOBS, F., KNOOP, C., PATTEET, S. & LAGROU, K. 2017. Culture-based methods and molecular tools for azole-resistant *Aspergillus fumigatus* detection in a Belgian University hospital. *J Clin Microbiol,* 55**,** 2391-2399.

MORIO, F., AUBIN, G. G., DANNER-BOUCHER, I., HALOUN, A., SACCHETTO, E., GARCIA-HERMOSO, D., BRETAGNE, S., MIEGEVILLE, M. & LE PAPE, P. 2012. High prevalence of triazole resistance in *Aspergillus fumigatus,* especially mediated by TR/L98H, in a French cohort of patients with cystic fibrosis. *Journal of Antimicrobial Chemotherapy,* 67**,** 1870-3.

MORTENSEN, K. L., JENSEN, R. H., JOHANSEN, H. K., SKOV, M., PRESSLER, T., HOWARD, S. J., LEATHERBARROW, H., MELLADO, E. & ARENDRUP, M. C. 2011a. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *Journal of Clinical Microbiology,* 49**,** 2243-51.

MORTENSEN, K. L., JOHANSEN, H. K., FUURSTED, K., KNUDSEN, J. D., GAHRN-HANSEN, B., JENSEN, R. H., HOWARD, S. J. & ARENDRUP, M. C. 2011b. A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. *European Journal of Clinical Microbiology and Infectious Diseases,* 30**,** 1355-1363.

MORTENSEN, K. L., MELLADO, E., LASS-FLORL, C., RODRIGUEZ-TUDELA, J. L., JOHANSEN, H. K. & ARENDRUP, M. C. 2010. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrobial Agents and Chemotherapy,* 54**,** 4545-9.

MULLINS, J., HARVEY, R. & SEATON, A. 1976. Sources and incidence of airborne *Aspergillus fumigatus* (*Fres*). *Clinical Allergy,* 6**,** 209-17.

MULLINS, J., HUTCHESON, P. S. & SLAVIN, R. G. 1984. *Aspergillus fumigatus* spore concentration in outside air: Cardiff and St Louis compared. *Clinical Allergy,* 14**,** 351-4.

MUSHI, M. F., BUNAME, G., BADER, O., GROSS, U. & MSHANA, S. E. 2016. *Aspergillus fumigatus* carrying TR34/L98H resistance allele causing complicated suppurative otitis media in Tanzania: call for improved diagnosis of fungi in sub-Saharan Africa. *BMC Infect Dis,* 16**,** 464.

MYERS, D. A., ISAZA, R., BEN-SHLOMO, G., ABBOTT, J. & PLUMMER, C. E. 2009. Fungal keratitis in a gopher tortoise (*Gopherus polyphemus*). *Journal of Zoo and Wildlife Medicine,* 40.

NAM, H. S., JEON, K., UM, S. W., SUH, G. Y., CHUNG, M. P., KIM, H., KWON, O. J. & KOH, W. J. 2010. Clinical characteristics and treatment outcomes of chronic necrotizing pulmonary aspergillosis: a review of 43 cases. *International Journal of Infectious Diseases,* 14**,** e479-82.

NARDONI, S., CECCHERELLI, R., ROSSI, G. & MANCIANTI, F. 2006. Aspergillosis in *Larus cachinnans micaellis*: survey of eight cases. *Mycopathologia,* 161**,** 317-321.

NASH, A. & RHODES, J. 2017. Simulations of *CYP51A* from *Aspergillus fumigatus* in a model bilayer provide insights into triazole drug resistance. *Medical Mycology*, myx056**,** 1-13.

NASH, G., IRVINE, R., KERSCHMANN, R. L. & HERNDIER, B. 1997. Pulmonary aspergillosis in acquired immune deficiency syndrome: autopsy study of an emerging

pulmonary complication of human immunodeficiency virus infection. *Human Pathology,* 28**,** 1268-75.

NAWROT, U., KURZYK, E., ARENDRUP, M. C., MROCZYNSKA, M., WLODARCZYK, K., SULIK-TYSZKA, B., WROBLEWSKA, M., USSOWICZ, M., ZDZIARSKI, P., NIEWINSKA, K. & BRILLOWSKA-DABROWSKA, A. 2018. Detection of Polish clinical *Aspergillus fumigatus* isolates resistant to triazoles. *Medical Mycology,* 56**,** 121-124.

NEGRI, C. E., GONÇALVES, S. S., XAFRANSKI, H., BERGAMASCO, M. D., AQUINO, V. R., CASTRO, P. T. & COLOMBO, A. L. 2014. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. *Journal of Clinical Microbiology,* 52**,** 3633-3640.

NEOFYTOS, D., HORN, D., ANAISSIE, E., STEINBACH, W., OLYAEI, A., FISHMAN, J., PFALLER, M., CHANG, C., WEBSTER, K. & MARR, K. 2009. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. *Clinical Infectious Diseases,* 48**,** 265-73.

NEUMANN, F. E. 2016. Aspergillosis in domesticated birds. *Journal of Comparative Pathology,* 155**,** 102-4.

OBAYASHI, T., NEGISHI, K., SUZUKI, T. & FUNATA, N. 2008. Reappraisal of the serum (1→3)-β-D-glucan assay for the diagnosis of invasive fungal infections—a study based on autopsy cases from 6 years. *Clinical Infectious Diseases,* 46**,** 1864-1870.

ODABASI, Z., MATTIUZZI, G., ESTEY, E., KANTARJIAN, H., SAEKI, F., RIDGE, R. J., KETCHUM, P. A., FINKELMAN, M. A., REX, J. H. & OSTROSKY-ZEICHNER, L. 2004. Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. *Clinical Infectious Diseases,* 39**,** 199-205.

OHBA, H., MIWA, S., SHIRAI, M., KANAI, M., EIFUKU, T., SUDA, T., HAYAKAWA, H. & CHIDA, K. 2012. Clinical characteristics and prognosis of chronic pulmonary aspergillosis. *Respiratory Medicine,* 106**,** 724-9.

OLIAS, P., HAUCK, R., WINDHAUS, H., VAN DER GRINTEN, E., GRUBER, A. D. & HAFEZ, H. M. 2010. Articular aspergillosis of hip joints in Turkeys *Avian Diseases,* 54**,** 1098-1101.

OSSENT, P. 1987. Systemic aspergillosis and mucormycosis in 23 cats. *Veterinary Record,* 120**,** 330-333.

OSTROSKY-ZEICHNER, L., ALEXANDER, B. D., H., K. D., VAZQUEZ, J., PAPPAS, P. G., SAEKI, F., KETCHUM, P. A., WINGARD, J., SCHIFF, R., TAMURA, H., FINKELMAN, M. A. & REX, J. H. 2005. Multicenter clinical evaluation of the (1→3) β-D-Glucan assay as an aid to diagnosis of fungal infections in humans. *Clinical Infectious Diseases,* 41**,** 654-659.

OZKUTUK, A., ERGON, C., METIN, D. Y., YUCESOY, M. & POLAT, S. H. 2008. Comparison of disk diffusion, E-test and broth microdilution test in determination of susceptibility of *Aspergillus* species to amphotericin B, itraconazole and voriconazole. *Journal of Chemotherapy,* 20**,** 87-92.

PAGE, I. D., RICHARDSON, M. & DENNING, D. W. 2015. Antibody testing in aspergillosis--quo vadis? *Medical Mycology,* 53**,** 417-39.

PAKES, S. P., NEW, A. E. & BENBROOK, S. C. 1967. Pulmonary aspergillosis in a cat. *Journal of the American Veterinary Medical Association,* 151**,** 950-953.

PAPPAS, P. G., ALEXANDER, B. D., ANDES, D. R., HADLEY, S., KAUFFMAN, C. A., FREIFELD, A., ANAISSIE, E. J., BRUMBLE, L. M., HERWALDT, L. A., ITO, J., KONTOYIANNIS, D. P., LYON, G. M., MARR, K. A., MORRISON, V. A., PARK, B. J., PATTERSON, T. F., PERL, T. M., OSTER, R. A., SCHUSTER, M. G., WALKER, R., WALSH, T. J., WANNEMUEHLER, K. A. & CHILLER, T. M. 2010. Invasive fungal

infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Clinical Infectious Diseases,* 50**,** 1101-1111.

PARIZE, P., CHANDESRIS, M-O., LANTERNIER, F., POIRÉE, S., VIARD, J-P., BIENVENU, B., MIMOUN, M., MÉCHAI, F., MAMZER, M-F., HERMAN, P., BOUGNOUX, M-E., LECUIT, M., LORTHOLARY, O. 2009. Antifungal therapy of *Asperigllus* invasive otitis externa: efficacy of voriconazole and review. *Antimicrobial Agents and Chemotherapy.* 53, 1048-1053.

PATTERSON, T. F., THOMPSON III, G. R., DENNING, D. W., FISHMAN, J. A., HADLEY, S., HERBRECHT, R., KONTOYIANNIS, D. P., MARR, K. A., MORRISON, V. A., HONG NGUYEN, M., SEGAL, B. H., STEINBACH, W. J., STEVENS, D. A., WALSH, T. J., WINGARD, J. R., YOUNG, J. A. H. & BENNETT, J. E. 2016. Practice guidelines for the diagnosis and management of Aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clinical Infectious Diseases,* 63**,** e1-e60.

PEETERS, D. & CLERCX, C. 2007. Update on canine sinonasal aspergillosis. *Veterinary Clinics of North America: Small Animal Practice,* 37**,** 901-916.

PEETERS, D., DAY, M. J. & CLERCX, C. 2005. An immunohistochemical study of canine nasal aspergillosis. *Journal of Comparative Pathology,* 132**,** 283-288.

PELÁEZ, T., ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., VALERIO, M., BLANCO, J. L., GARCIA, M. E., MUÑOZ, P., CUENCA-ESTRELLA, M. & BOUZA, E. 2013. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. *Journal of Medical Microbiology,* 62**,** 474-478.

PELÁEZ, T., GIJON, P., BUNSOW, E., BOUZA, E., SANCHEZ-YEBRA, W., VALERIO, M., GAMA, B., CUENCA-ESTRELLA, M. & MELLADO, E. 2012. Resistance to voriconazole due to a G448S substitution in *Aspergillus fumigatus* in a patient with cerebral aspergillosis. *Journal of Clinical Microbiology,* 50**,** 2531-4.

PELÁEZ, T., MONTEIRO, M. C., GARCIA-RUBIO, R., BOUZA, E., GOMEZ-LOPEZ, A. & MELLADO, E. 2015. First detection of *Aspergillus fumigatus* azole-resistant strain due to *Cyp51A* TR46/Y121F/T289A in an azole-naive patient in Spain. *New Microbes and New*

Infections, 6**,** 33-4.

PEREZ, J., GARCIA, P. M., MENDEZ, A., ASTORGA, R., LUQUE, I. & TARRADAS, C. 2003. Outbreak of aspergillosis in a flock of adult ostriches (*Struthio camelus*). *Veterinary Record,* 153**,** 124-125.

PÉREZ, V., CORPA, J. M., F., G. M. J., ADÚRIZ, J. J. & JENSEN, H. E. 1999. Generalized aspergillosis in dairy sheep. *Zentralbl Veterinarmed B,* 46**,** 613-621.

PÉREZ, V., CORPA, J. M., GARCIA MARIN, J. F., ADURIZ, J. J. & JENSEN, H. E. 1998. Mammary and systemic aspergillosis in dairy sheep. *Veterinary Pathology,* 35**,** 235-240.

PERVEEN, I., SEHAR, S., NAZ, I. & AHMED, S. Prospective evaluation of azole resistance in *Aspergillus fumigatus* clinical isolates in Pakistan. Seventh Advances Against Aspergillosis conference, 3-5 March 2016 2016 Manchester, UK.

PFALLER, M. A., DIEKEMA, D. J., GHANNOUM, M. A., REX, J. H., ALEXANDER, B. D., ANDES, D., BROWN, S. D., CHATURVEDI, V., ESPINEL-INGROFF, A., FOWLER, C. L., JOHNSON, E. M., KNAPP, C. C., MOTYL, M. R., OSTROSKY-ZEICHNER, L., SHEEHAN, D. J., WALSH, T. J. & SUBCOMMITTEE., C. L. S. I. A. T. 2009. Wild-type MIC distribution and epidemiological cutoff values for *Aspergillus fumigatus* and three triazoles as determined by the Clinical and Laboratory Standards Institute broth microdilution methods. *Journal of Clinical Microbiology,* 47**,** 3142-6.

PHALEN, D. N. 2000. Respiratory medicine of cage and aviary birds. *Veterinary Clinics of North America: Exotic Animal Practice,* 3**,** 423-52, vi.

PHAM, C. D., REISS, E., HAGEN, F., MEIS, J. F. & LOCKHART, S. R. 2014. Passive surveillance for azole-resistant *Aspergillus fumigatus*, United States, 2011-2013. *Emerging Infectious Diseases,* 20**,** 1498-503.

PICKERING, J. W., SANT, H. W., BOWLES, C. A. P., ROBERTS, W. L. & WOODS, G. L. 2005. Evaluation of a (1→3)-β-d-Glucan assay for diagnosis of invasive fungal infections. *Journal of Clinical Microbiology,* 43**,** 5957-5962.

PICKETT, J. P., MOORE, C. P., BEEHLER, B. A., GENDRON-FITZPATRICK, A. & DUBIELZIG, R. R. 1985. Bilateral chorioretinitis secondary to disseminated aspergillosis in an alpaca. *Journal of the American Veterinary Medical Association,* 187**,** 1241-1243.

PITT, J. I. 1994. The current role of *Aspergillus* and *Penicillium* in human and animal health. *Journal of Medical and Veterinary Mycology,* 32 Suppl 1**,** 17-32.

POSTERARO, B., MATTEI, R., TRIVELLA, F., MAFFEI, A., TORRE, A., DE CAROLIS, E., POSTERARO, P., FADDA, G. & SANGUINETTI, M. 2011. Uncommon *Neosartorya udagawae* fungus as a causative agent of severe corneal infection. *J Clin Microbiol,* 49**,** 2357-2360.

POWERS-FLETCHER, M. V. & HANSON, K. E. 2016. Molecular diagnostic testing for *Aspergillus*. *Journal of Clinical Microbiology,* 54**,** 2655-2660.

PRAHL, S., JEPSON, P. D., SANCHEZ-HANKE, M., DEAVILLE, R. & SIEBERT, U. 2011. Aspergillosis in the middle ear of a harbour porpoise (*Phocoena phocoena*): a case report. *Mycoses,* 54**,** e260-e264.

PRICE, C. L., PARKER, J. E., WARRILOW, A. G., KELLY, D. E. & KELLY, S. L. 2015. Azole fungicides - understanding resistance mechanisms in agricultural fungal pathogens. *Pest Management Science,* 71**,** 1054-8.

PRIGITANO, A., VENIER, V., COGLIATI, M., DE LORENZIS, G., ESPOSTO, M. C. & TORTORANO, A. M. 2014. Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012. *Euro Surveillance,* 19**,** pii=20747.

PUNTENNEY, S. B., WANG, Y. & FORSBERG, N. E. Mycotic infections in livestock: Recent insights and studies on etiology,diagnostics and prevention of Hemorrhagic Bowel Syndrome. *In:* ARIZONA, U. O., ed. Southwest Nutrition & Management Conference, 2003 Phoenix, Tucson. 49-63.

RATH, P. M., BUCHHEIDT, D., SPIESS, B., ARFANIS, E., BUER, J. & STEINMANN, J. 2012. First reported case of azole-resistant *Aspergillus fumigatus* due to the TR/L98H mutation in Germany. *Antimicrobial Agents and Chemotherapy,* 56**,** 6060-1.

REDIG, P. T. Diagnosis of avian aspergillosis. *In:* KORNELSEN, M. J., ed. Annual Conference of the Association of Avian Veterinarians, 1994 Reno, NV. 335.

REIDARSON, T. H., HARRELL, J. H., RINALDI, M. G. & MCBAIN, J. 1998. Bronchoscopic and serologic diagnosis of *Aspergillus fumigatus* pulmonary infection in a bottlenose dolphin (*Tursiops truncatus*). *Journal of Zoo and Wildlife Medicine,* 29.

REIDARSON, T. H., MCBAIN, J. F. & DALTON, L. M. 1999. Diagnosis and treatment of fungal infections in marine mammals. *In:* FOWLER, M. E. & MILLER, R. E. (eds.) *Zoo and wildlife animal medicine: current therapy.* Fourth ed. St Louis, Missouri: Saunders.

REN, J., JIN, X., ZHANG, Q., ZHENG, Y., LIN, D. & YU, Y. 2017. Fungicides induced triazole-resistance in *Aspergillus fumigatus* associated with mutations of TR46/Y121F/T289A and its appearance in agricultural fields. *J Hazard Mater,* 326**,** 54-60.

RODRIGUEZ-TUDELA, J. L., ALCAZAR-FUOLI, L., MELLADO, E., ALASTRUEY-IZQUIERDO, A., MONZON, A. & CUENCA-ESTRELLA, M. 2008. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy,* 52**,** 2468-72.

RUSSCHER, A., VAN PAASSEN, J., DOFFERHOFF, P. A. & KUIJPER, E. J. 2014. High azole resistance rate of *Aspergillus fumigatus* at intensive care unit in a Dutch tertiary hospital. *Nederlands Tijdschrift voor Medische Microbiologie,* 22**,** 121.

RUSSELL, P. E. 2005. A century of fungicide evolution. *The Journal of Agricultural Science,* 143**,** 11-25.

SAMSON, R. A., HONG, S., PETERSON, S. W., FRISVAD, J. C. & VARGA, J. 2007. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Studies in Mycology,* 59**,** 147-203.

SANSOM, J., FEATHERSTONE, H. & BARNETT, K. C. 2005. Keratomycosis in six horses in the United Kingdom. *The Veterinary Record,* 156**,** 13-17.

SANTORO, K., MATIC, S., GISI, U., SPADARO, D., PUGLIESE, M. & GULLINO, M. L. 2017. Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* isolates from organic substrates with special emphasis on compost. *Pest Manag Sci,* 73**,** 2481-2494.

SATO, Y. & ITAGAKI, T. 2010. Fungal airsacculitis associated with multiple helminth infestations in a black-eared kite (*Milvus migrans*). *Avian Diseases,* 54**,** 965-968.

SAUNDERS, J. H., CLERCX, C., SNAPS, F. R., SULLIVAN, M., DUCHATEAU, L., VAN BREE, H. J. & DONDELINGER, R. E. 2004. Radiographic, magnetic resonance imaging, computed tomographic, and rhinoscopic features of nasal aspergillosis in dogs. *Journal of the American Veterinary Medical Association,* 225**,** 1703-12.

SAUNDERS, J. H. & VAN BREE, H. 2003. Comparison of radiography and computed tomography for the diagnosis of canine nasal aspergillosis. *Veterinary Radiology and Ultrasound,* 44**,** 414-9.

SAUTTER, J. H., STEELE, D. S. & HENRY, J. F. 1955. Symposium on granulomatous diseases II. *Journal of the American Veterinary Medical Association,* 127**,** 518.

SCHAUWVLIEGHE, A., VONK, A. G., BUDDINGH, E. P., HOEK, R. A. S., DALM, V. A., KLAASSEN, C. H. W. & RIJNDERS, B. J. A. 2017. Detection of azole-susceptible and azole-resistant *Aspergillus* coinfection by *cyp51A* PCR amplicon melting curve analysis. *J Antimicrob Chemother,* 72**,** 3047-3050.

SCHIEFER, B. 1965. Zur histopahtologie der durch *Candida-*, *Aspergillus-* und *Mucor-Arten* verusachten darmmykosen bei katzen mit panleukopenie. *Dtsch Tierarztl Wochenschr,* 72**,** 73-76.

SCHULTZ, R. M., JOHNSON, E. G., WISNER, E. R., BROWN, N. A., BYRNE, B. A. & SYKES, J. E. 2008. Clinicopathologic and diagnostic imaging characteristics of

systemic aspergillosis in 30 dogs. *Journal of Veterinary Internal Medicine,* 22**,** 851-859.

SCOTT, H. H. 1930. Tuberculosis in man and lower animals: a study in comparative pathology.

In: MRC (ed.) *Special Report Series.* London, UK: Medical Research Council.

SEGAL, B. H. & ROMANI, L. R. 2009. Invasive aspergillosis in chronic granulomatous disease. *Medical Mycology,* 47**,** S282-90.

SEVERO, L. C., BOHRER, J. C., GEYER, G. R. & FERREIRO, L. 1989. Invasive aspergillosis in an alpaca (*Lama pacos*). *Journal of Medical and Veterinary Mycology,* 27**,** 193-195.

SEYEDMOUSAVI, S., GUILLOT, J., ARNE, P., DE HOOG, G. S., MOUTON, J. W., MELCHERS, W. J. & VERWEIJ, P. E. 2015. *Aspergillus* and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease. *Medical Mycology,* 53**,** 765-97.

SEYEDMOUSAVI, S., HASHEMI, S. J., ZIBAFAR, E., ZOLL, J., HEDAYATI, M. T., MOUTON, J. W., MELCHERS, W. J. G. & VERWEIJ, P. E. 2013. Azole-resistant *Aspergillus fumigatus*, Iran. *Emerging Infectious Diseases,* 19**,** 832-833.

SHALHOUB, S., LUONG, M. L., HOWARD, S. J., RICHARDSON, S., SINGER, L. G., CHAPARRO, C., KESHAVJEE, S., AKINLOLU, Y., ROTSTEIN, C., MAZZULLI, T. & HUSAIN, S. 2015. Rate of *cyp51A* mutation in *Aspergillus fumigatus* among lung transplant recipients with targeted prophylaxis. *Journal of Antimicrobial Chemotherapy,* 70**,** 1064-7.

SHARMA, C., HAGEN, F., MOROTI, R., MEIS, J. F. & CHOWDHARY, A. 2015. Triazole-resistant *Aspergillus fumigatus* harbouring G54 mutation: is it de novo or environmentally acquired? *Journal of Global Antimicrobial Resistance,* 3**,** 69-74.

SHARMAN, M., PAUL, A., DAVIES, D., MACKAY, B., SWINNEY, G., BARRS, V., ARTEAGA, A., ROBERTSON, I. D. & MANSFIELD, C. 2010. Multi-centre assessment of mycotic rhinosinusitis in dogs: a retrospective study of initial treatment success (1998 to 2008). *Journal of Small Animal Practice,* 51**,** 423-427.

SHARP, N. J. H., HARVEY, C. E. & SULLIVAN, M. 1991. Canine nasal aspergillosis and penicilliosis. *Compendium on Continuing Education for the Practising Veterinarian,* 13**,** 41-49.

SHAUKAT, A., BAKRI, F., YOUNG, P., HAHN, T., BALL, D., BAER, M. R., WETZLER, M., SLACK, J. L., LOUD, P., CZUCZMAN, M., MCCARTHY, P. L., WALSH, T. J. & SEGAL, B. H. 2005. Invasive filamentous fungal infections in allogeneic hematopoietic stem cell transplant recipients after recovery from neutropenia: clinical, radiologic, and pathologic characteristics. *Mycopathologia,* 159**,** 181-8.

SHENG, C., MIAO, Z., JI, H., YAO, J., WANG, W., CHE, X., DONG, G., LÜ, J., GUO, W. & ZHANG, W. 2009. Three-dimensional model of lanosterol 14 alpha-demethylase from *Cryptococcus neoformans*: active-site characterization and insights into azole binding. *Antimicrobial Agents and Chemotherapy,* 53**,** 3487-3495.

SIMPSON, V. 2011. Spinal aspergillosis in pheasants. *The Veterinary Record,* 169**,** 562.

SMITH, L. N. & HOFFMAN, S. B. 2010. A case series of unilateral orbital aspergillosis in three cats and treatment with voriconazole. *Veterinary Opthalmology,* 13**,** 190-203.

SNELDERS, E., CAMPS, S. M., KARAWAJCZYK, A., SCHAFTENAAR, G., KEMA, G. H., VAN DER LEE, H. A., KLAASSEN, C. H., MELCHERS, W. J. & VERWEIJ, P. E. 2012. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS One,* 7**,** e31801.

SNELDERS, E., HUIS IN 'T VELD, R. A., RIJS, A. J. M., KEMA, G. H. J., MELCHERS, W. J. G. & VERWEIJ, P. E. 2009. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazole. *Applied and Environmental Microbiology,* 75**,** 4053-4057.

SNELDERS, E., KARAWAJCZYK, A., SCHAFTENAAR, G., VERWEIJ, P. E. & MELCHERS, W. J. 2010. Azole resistance profile of amino acid changes in *Aspergillus fumigatus CYP51A* based on protein homology modeling. *Antimicrobial Agents and Chemotherapy,* 54**,** 2425-2430.

SNELDERS, E., VAN DER LEE, H. A., KUIJPERS, J., RIJS, A. J., VARGA, J., SAMSON, R. A., MELLADO, E., DONDERS, A. R., MELCHERS, W. J. & VERWEIJ, P. E. 2008. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Medicine,* 5**,** e219.

SOUTHARD, C. 1987. Bronchopulmonary aspergillosis in a dog. *Journal of the American Veterinary Medical Association,* 190**,** 875-877.

STEINMANN, J., HAMPRECHT, A., VEHRESCHILD, M. J., CORNELY, O. A., BUCHHEIDT, D., SPIESS, B., KOLDEHOFF, M., BUER, J., MEIS, J. F. & RATH, P. M. 2015. Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. *Journal of Antimicrobial Chemotherapy,* 70**,** 1522-6.

STENGEL, C. Meticulous debridement as sole management for successful outcome in 6 dogs with sinonasal aspergillosis (SNA). 27th ECVIM-CA Congress, 14-16 September 2017 St. Julian's, Malta.

STENSVOLD, C. R., JØRGENSEN, L. N. & ARENDRUP, M. C. 2012. Azole-resistant invasive aspergillosis: relationship to agriculture. *Current Fungal Infection Reports,* 6**,** 178-191.

STERGIOPOULOU, T., MELETIADIS, J., ROILIDES, E., KLEINER, D. E., SCHAUFELE, R., RODEN, M., HARRINGTON, S., DAD, L., SEGAL, B. & WALSH, T. J. 2007. Host-dependent patterns of tissue injury in invasive pulmonary aspergillosis. *American Journal of Clinical Pathology,* 127**,** 349-55.

STOKES, R. 1973. Letter: intestinal mycosis in a cat. *Austraian Veterinary Journal,* 49**,** 499-500.

SUEDMEYER, W. K., BERMUDEZ, A. J. & FALES, W. H. 2002. Treatment of epidermal cysts associated with *Aspergillus fumigatus* and *Alternaria* species in a silky bantam chicken *Journal of Avian Medicine and Surgery,* 16**,** 133-137.

SUGUI, J. A., PETERSON, S. W., FIGAT, A., HANSEN, B., SAMSON, R. A., MELLADO, E., CUENCA-ESTRELLA, M. & KWON-CHUNG, K. J. 2014. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J Clin Microbiol,* 52**,** 3707-3721.

SUGUI, J. A., VINH, D. C., NARDONE, G., SHEA, Y. R., CHANG, Y. C., ZELAZNY, A. M., MARR, K. A., HOLLAND, S. M. & KWON-CHUNG, K. J. 2010. *Neosartorya udagawae* (*Aspergillus udagawae*), an emerging agent of aspergillosis: how different is it from *Aspergillus fumigatus*? *Journal of Clinical Microbiology,* 48**,** 220-228.

SUTTON, D. A., SUMMERBELL, R. C., SAMSON, R. & RINALDE, M. F. First report of *Neosartorya spinosa* (Raper&Fennell)Kozakiewicz inciting human disease. American Society for Microbiology General Meeting, 2002 Salt Lake City, UT. 212.

TAMBORINI, A., ROBERTSON, E., TALBOT, J. J. & BARRS, V. R. 2016. Sinonasal aspergillosis in a British Shorthair cat in the UK. *Journal of Feline Medicine and Surgery Open Reports,* 2**,** 2055116916653775.

TANGWATTANACHULEEPORN, M., MINARIN, N., SAICHAN, S., SERMSRI, P., MITKORNBUREE, R., GROß, U., CHINDAMPORN, A. & BADER, O. 2017. Prevalence of azole-resistant *Aspergillus fumigatus* in the environment of Thailand. *Medical Mycology,* 55**,** 429-435.

TAPPE, J. P., CHANDLER, F. W., LIU, S. K. & DOLENSEK, E. P. 1984. Aspergillosis in two San Esteban chuckwallas. *Journal of the American Veterinary Medical Association,* 185**,** 1425-1428.

TASHIRO, M. & IZUMIKAWA, K. 2016. The current status of drug-resistant *Aspergillus*. *Medical Mycology Journal,* 57**,** J103-J12.

TASHIRO, M., IZUMIKAWA, K., HIRANO, K., IDE, S., MIHARA, T., HOSOGAYA, N., TAKAZONO, T., MORINAGA, Y., NAKAMURA, S., KURIHARA, S., IMAMURA, Y., MIYAZAKI, T., NISHINO, T., TSUKAMOTO, M., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., YASUOKA, A., TASHIRO, T. & KOHNO, S. 2012a. Correlation between triazole treatment history and susceptibility in clinically isolated *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy,* 56**,** 4870-5.

TASHIRO, M., IZUMIKAWA, K., MINEMATSU, A., HIRANO, K., IWANAGA, N., IDE, S., MIHARA, T., HOSOGAYA, N., TAKAZONO, T., MORINAGA, Y., NAKAMURA, S.,

KURIHARA, S., IMAMURA, Y., MIYAZAKI, T., NISHINO, T., TSUKAMOTO, M., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., YASUOKA, A., TASHIRO, T. & KOHNO, S. 2012b. Antifungal Susceptibilities of *Aspergillus fumigatus* Clinical Isolates Obtained in Nagasaki, Japan. *Antimicrobial Agents and Chemotherapy,* 56**,** 584-587.

TASKER, S., KNOTTENBELT, C. M., MUNRO, E. A. C., STONEHEWER, J., SIMPSON, J. W. & MACKIN, A. J. 1999. Aetiology and diagnosis of persistent nasal disease in the dog: a retrospective study of 42 cases. *The Journal of Small Animal Practice,* 40**,** 473-478.

TAYLOR, A., PETERS, I., DHAND, N. K., WHITNEY, J., JOHNSON, L. R., BEATTY, J. A. & BARRS, V. R. 2016. Evaluation of serum *Aspergillus*-specific Immunoglobulin A by indirect ELISA for diagnosis of feline upper respiratory tract aspergillosis. *Journal of Veterinary Internal Medicine,* 30**,** 1708-1714.

TELL, L. A. 2005. Aspergillosis in mammals and birds: impact on veterinary medicine. *Medical Mycology,* 43**,** 71-73.

THRONE STEINLAGE, S. J., SANDER, J. E., BROWN, T. P., LOBSINGER, C. M., THAYER, S. G. & MARTINEZ, A. 2003. Disseminated mycosis in layer cockerels and pullets. *Avian Diseases,* 47**,** 229-233.

TOMSA, K., GLAUS, T. M., ZIMMER, C. & GREENE, C. E. 2003. Fungal rhinitis and sinusitis in three cats. *Journal of the American Veterinary Medical Association,* 222**,** 1380-4, 1365.

TOSKOVA, M., PALOUSOVA, D., KOCMANOVA, I., PAVLOVSKY, Z., TIMILSINA, S., LENGEROVA, M., MAYER, J. & RACIL, Z. 2013. Invasive mould disease involving the gastrointestinal tract caused by *Neosartorya pseudofischeri* in a haematological patient. *Mycoses,* 56**,** 385-8.

TOYOTOME, T., FUJIWARA, T., KIDA, H., MATSUMOTO, M., WADA, T. & KOMATSU, R. 2016. Azole susceptibility in clinical and environmental isolates of *Aspergillus fumigatus* from eastern Hokkaido, Japan. *Journal of Infection and Chemotherapy,* 22**,** 648-50.

TOYOTOME, T., HAGIWARA, D., KIDA, H., OGI, T., WATANABE, A., WADA, T., KOMATSU, R. & KAMEI, K. 2017. First clinical isolation report of azole-resistant *Aspergillus fumigatus* with TR34/L98H-type mutation in Japan. *Journal of Infection and Chemotherapy,* 23**,** 579-581.

TSAI, S. S., PARK, J. H., HIRAI, K. & ITAKURA, C. 1992. Aspergillosis and candidiasis in psittacine and passeriforme birds with particular reference to nasal lesions. *Avian Pathology,* 21**,** 699-709.

ULLMANN, A. J., AGUADO, J. M., ARIKAN-AKDAGLI, S., DENNING, D. W., GROLL, A. H., LAGROU, K., LASS-FLORL, C., LEWIS, R. E., MUNOZ, P., VERWEIJ, P. E., WARRIS, A., ADER, F., AKOVA, M., ARENDRUP, M. C., BARNES, R. A., BEIGELMAN-AUBRY, C., BLOT, S., BOUZA, E., BRUGGEMANN, R. J. M., BUCHHEIDT, D., CADRANEL, J., CASTAGNOLA, E., CHAKRABARTI, A., CUENCA-ESTRELLA, M., DIMOPOULOS, G., FORTUN, J., GANGNEUX, J. P., GARBINO, J., HEINZ, W. J., HERBRECHT, R., HEUSSEL, C. P., KIBBLER, C. C., KLIMKO, N., KULLBERG, B. J., LANGE, C., LEHRNBECHER, T., LOFFLER, J., LORTHOLARY, O., MAERTENS, J., MARCHETTI, O., MEIS, J. F., PAGANO, L., RIBAUD, P., RICHARDSON, M., ROILIDES, E., RUHNKE, M., SANGUINETTI, M., SHEPPARD, D. C., SINKO, J., SKIADA, A., VEHRESCHILD, M., VISCOLI, C. & CORNELY, O. A. 2018. Diagnosis and management of *Aspergillus* diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline. *Clinical Microbiology and Infection,* 24 Suppl 1**,** e1-e38.

URI, N., COHEN-KEREM, R., ELMALAH, I., DOWECK, I. & GREENBER, E. 2003. Classification of fungal sinusitis in immunocompetent patients. *Otolaryngology - Head and Neck Surgery,* 129**,** 372-378.

VAN DER LINDEN, J. W., ARENDRUP, M. C., WARRIS, A., LAGROU, K., PELLOUX, H., HAUSER, P. M., CHRYSSANTHOU, E., MELLADO, E., KIDD, S. E., TORTORANO, A. M., DANNAOUI, E., GAUSTAD, P., BADDLEY, J. W., UEKOTTER, A., LASS-FLORL, C., KLIMKO, N., MOORE, C. B., DENNING, D. W., PASQUALOTTO, A. C., KIBBLER, C., ARIKAN-AKDAGLI, S., ANDES, D., MELETIADIS, J., NAUMIUK, L., NUCCI, M., MELCHERS, W. J. & VERWEIJ, P. E. 2015. Prospective multicenter international

surveillance of azole resistance in *Aspergillus fumigatus*. *Emerging Infectious Diseases,* 21**,** 1041-4.

VAN DER LINDEN, J. W., CAMPS, S. M., KAMPINGA, G. A., ARENDS, J. P., DEBETS-OSSENKOPP, Y. J., HAAS, P. J., RIJNDERS, B. J., KUIJPER, E. J., VAN TIEL, F. H., VARGA, J., KARAWAJCZYK, A., ZOLL, J., MELCHERS, W. J. & VERWEIJ, P. E. 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clinical Infectious Diseases,* 57**,** 513-520.

VAN DER LINDEN, J. W. M., ARENDRUP, M. C., VAN DER LEE, H. A. L., MELCHERS, W. J. G. & VERWEIJ, P. E. 2009. Azole containing agar plates as a screening tool for azole resistance of *Aspergillus fumigatus*. *Mycoses,* 52**,** 19.

VAN DER LINDEN, J. W. M., SNELDERS, E., KAMPINGA, G. A., RIJNDERS, B. J. A., MATTSSON, E., DEBETS-OSSENKOPP, Y. J., KUIJPER, E. J., VAN TIEL, F. H., MELCHERS, W. J. G. & VERWEIJ, P. E. 2011. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. *Emerging Infectious Diseases,* 17**,** 1846-1854.

VAN VEEN, L., DWARS, R. M. & FABRI, T. H. F. 1999. Mycotic spondylitis in broilers caused by *Aspergillus fumigatus* resulting in partial anterior and posterior paralysis. *Avian Pathology,* 28**,** 487-490.

VANDEN BOSSCHE, H., MACKENZIE, D. W. R. & CAUWENBERGH, G. 1988. *Aspergillus and aspergillosis,* New York, N. Y., Plenium Press.

VEDRINE, B. & FRIBOURG-BLANC, L. A. 2018. Treatment of sinonasal aspergillosis by debridement and sinonasal deposition therapy with clotrimazole under rhinoscopic guidance. *Journal of the American Animal Hospital Association,* 54.

VERMEULEN, E., LAGROU, K. & VERWIEJ, P. E. 2013. Azole resistance in *Aspergillus fumigatus*; a growing public health concern. *Current Opinion in Infectious Diseases,* 26**,** 493-500.

VERMEULEN, E., MAERTENS, J., SCHOEMANS, H. & LAGROU, K. 2012. Azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation emerging in Belgium. *Euro Surveillance,* 17**,** pii=20326.

VERWEIJ, P., MELLADO, E. & MELCHERS, W. J. G. 2007. Multiple-triazole–resistant aspergillosis. *The New England Journal of Medicine,* 356**,** 1481-1483.

VERWEIJ, P. E., ANANDA-RAJAH, M., ANDES, D., ARENDRUP, M. C., BRUGGEMANN, R. J., CHOWDHARY, A., CORNELY, O. A., DENNING, D. W., GROLL, A. H., IZUMIKAWA, K., KULLBERG, B. J., LAGROU, K., MAERTENS, J., MEIS, J. F., NEWTON, P., PAGE, I., SEYEDMOUSAVI, S., SHEPPARD, D. C., VISCOLI, C., WARRIS, A. & DONNELLY, J. P. 2015. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. *Drug Resistance Updates,* 21-22**,** 30-40.

VERWEIJ, P. E., HOWARD, S. J., MELCHERS, W. J. & DENNING, D. W. 2009a. Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. *Drug Resistance Updates,* 12**,** 141-7.

VERWEIJ, P. E., MENSINK, M., RIJS, A. J. M. M., DONNELLY, J. P., MEIS, J. F. G. M. & DENNING, D. W. 1998. In-vitro activities of amphotericin B, itraconazole and voriconazole

against 150 clinical and environmental *Aspergillus fumigatus* isolates. *Journal of Antimicrobial Chemotherapy,* 42**,** 389-392.

VERWEIJ, P. E., SNELDERS, E., KEMA, G. H. J., MELLADO, E. & MELCHERS, W. J. G. 2009b. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? . *The Lancet Infectious Diseases,* 9**,** 789-795.

VERWEIJ, P. E., TE DORSTHORST, D. T. A., RIJS, A. J. M. M., DE VRIES-HOSPERS, H. G. & MEIS, J. F. G. M. 2002. Nationwide Survey of In Vitro Activities of Itraconazole and Voriconazole against Clinical *Aspergillus fumigatus* Isolates Cultured between 1945 and 1998. *Journal of Clinical Microbiology,* 40**,** 2648-2650.

VERWEIJ, P. E., ZHANG, J., DEBETS, A. J. M., MEIS, J. F., VAN DE VEERDONK, F. L., SCHOUSTRA, S. E., ZWAAN, B. J. & MELCHERS, W. J. G. 2016. In-host adaptation and acquired triazole resistance in *Aspergillus fumigatus*: a dilemma for clinical management. *The Lancet Infectious Diseases,* 16**,** e251-e260.

VESTAPPEN, F. A. L. M. & DORRESTEIN, G. M. 2005. Aspergillosis in Amazon parrots after corticosteroid therapy for smoke-inhalation injury. *Journal of Avian Medicine and Surgery,* 19**,** 138-141.

VINH, D. C., SHEA, Y. R., JONES, P. A., FREEMAN, A. F., ZELAZNY, A. & HOLLAND, S. M. 2009a. Chronic invasive aspergillosis caused by *Aspergillus viridinutans*. *Emerg Infect Dis.,* 15**,** 1292-1294.

VINH, D. C., SHEA, Y. R., SUGUI, J. A., PARRILLA-CASTELLAR, E. R., FREEMAN, A. F., CAMPBELL, J. W., PITTALUGA, S., JONES, P. A., ZELAZNY, A., KLEINER, D., KWON-CHUNG, K. J. & HOLLAND, S. M. 2009b. Invasive aspergillosis due to *Neosartorya udagawae*. *Clinical Infectious Diseases,* 49**,** 102-111.

VOGLER, G. A. & WAGNER, J. E. 1975. What's your diagnosis. *Laboratory Animals,* 5**,** 14.

WADA, S., HOBO, S., ODE, H., NIWA, H. & MORIYAMA, H. 2013. Equine keratomycosis in Japan. *Veterinary Opthalmology,* 16**,** 1-9.

WALSH, T. J., ANAISSIE, E. J., DENNING, D. W., HERBRECHT, R., KONTOYIANNIS, D. P., MARR, K. A., MORRISON, V. A., SEGAL, B. H., STEINBACH, W. J., STEVENS, D. A., VAN BURIK, J. A., WINGARD, J. R., PATTERSON, T. F. & INFECTIOUS DISEASES SOCIETY OF AMERICA 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clinical Infectious Diseases,* 46**,** 327-60.

WANG, D. Y., GRICOURT, M., ARNE, P., THIERRY, S., SEGUIN, D., CHERMETTE, R., HUANG, W. Y., DANNAOUI, E., BOTTEREL, F. & GUILLOT, J. 2014. Mutations in the *Cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* isolated from avian farms in France and China. *Poultry Science,* 93**,** 12-5.

WEBER, M., SCHAER, J., WALTHER, G., KAERGER, K., STEINMANN, J., RATH, P., SPIESS, B., BUCHHEIDT, D., HAMPRECHT, A. & KURZAI, O. 2017. FunResDB-A web resource for genotypic susceptibility testing of *Aspergillus fumigatus*. *Medical Mycology*.

WEILAND, F. 1970. Intestinal mycosis in a cat. *Dtsch Tierarztl Wochenschr,* 77**,** 232-233.

WHITE, P. L., POSSO, R. B. & BARNES, R. A. 2017. Analytical and clinical evaluation of the PathoNostics AsperGenius assay for detection of invasive aspergillosis and resistance to azole antifungal drugs directly from plasma samples. *Journal of Clinical Microbiology,* 55**,** 2356-2366.

WHITE, T. J., BRUNS, T. & LEE, S. J. W. T. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* M. A. INNIS, D. H. G., J. J. SNINSKY & T. J. WHITE, (ed.) *PCR Protocols. A Guide to Methods and Applications.* New York: Academic Press.

WHITNEY, B. L., BROUSSARD, J. & STEFANACCI, J. D. 2005. Four cats with fungal rhinitis. *Journal of Feline Medicine and Surgery* 7**,** 53-58.

WIEDERHOLD, N. P., GIL, V. G., GUTIERREZ, F., LINDNER, J. R., ALBATAINEH, M. T., MCCARTHY, D. I., SANDERS, C., FAN, H., FOTHERGILL, A. W. & SUTTON, D. A. 2016. First detection of TR34 L98H and TR46 Y121F T289A *Cyp51* mutations in *Aspergillus fumigatus* isolates in the United States. *Journal of Clinical Microbiology,* 54**,** 168-71.

WILLIS, A. M., MARTIN, C. L. & STILES, J. 1999. Sino-orbital aspergillosis in a dog. *Journal of the American Veterinary Medical Association,* 214**,** 1644-7, 1639.

WOODS, R., BLYDE, D. J., SEAMAN, J. T. & THORNE, A. H. 1999. Fungal pneumonia in a captive black rhinoceros. *Australian Veterinary Journal,* 77**,** 717-9.

WU, C. J., WANG, H. C., LEE, J. C., LO, H. J., DAI, C. T., CHOU, P. H., KO, W. C. & CHEN, Y. C. 2015. Azole-resistant *Aspergillus fumigatus* isolates carrying TR34/L98H mutations in Taiwan. *Mycoses,* 58**,** 544-9.

XAVIER, M. O., SOARES, M. P., MEINERZ, A. R. M., NOBREI, M. O., OSÓRIO, L. G., DA SILVA FILHO, R. P. & MEIRELES, M. C. A. 2007. Aspergillosis: a limiting factor during recovery of captive magellanic penguins. *Brazilian Journal of Microbiology,* 38**,** 480-484.

XIAO, L., MADISON, V., CHAU, A. S., LOEBENBERG, D., PALERMO, R. E. & MCNICHOLAS, P. M. 2004. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14α-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. *Antimicrobial Agents and Chemotherapy,* 48**,** 568-574.

ZAFRA, R., PÉREZ, J., PÉREZ-ECIJA, R. A., BORGE, C., BUSTAMANTE, R., CARBONERO, A. & TARRADAS, C. 2008. Concurrent aspergillosis and ascites with high mortality in a farm of growing broiler chickens. *Avian Diseases,* 52**,** 711-713.

ZHANG, J., SNELDERS, E., ZWAAN, B. J., SCHOUSTRA, S. E., MEIS, J. F., VAN DIJK, K., HAGEN, F., VAN DER BEEK, M. T., KAMPINGA, G. A., ZOLL, J., MELCHERS, W. J. G., VERWEIJ, P. E. & DEBETS, A. J. M. 2017. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *mBio,* 8**,** e00791-17.

ZHANG, S., CORAPI, W., QUIST, E., GRIFFIN, S. & ZHANG, M. 2012. *Aspergillus versicolor*, a new causative agent of canine disseminated aspergillosis. *Journal of Clinical Microbiology,* 50**,** 187-91.

ZHAO, L., LIU, D., ZHANG, Q., ZHANG, S., WAN, J. & XIAO, W. 2007. Expression and homology modeling of sterol 14alpha-demethylase from *Penicillium digitatum*. *FEMS Microbiology Letters,* 277**,** 37-43.

ZHAO, Y., GARNAUD, C., BRENIER-PINCHART, M. P., THIEBAUT-BERTRAND, A., SAINT-RAYMOND, C., CAMARA, B., HAMIDFAR, R., COGNET, O., MAUBON, D., CORNET, M. & PERLIN, D. S. 2016. Direct molecular diagnosis of aspergillosis and *CYP51A* profiling from respiratory samples of French patients. *Frontiers in Microbiology,* 7**,** 1164.

ZIOLKOWSKA, G., TOKARZEWSKI, S. & NOWAKIEWICZ, A. 2014. Drug resistance of *Aspergillus fumigatus* strains isolated from flocks of domestic geese in Poland. *Poultry Science,* 93**,** 1106-12.

ZONDERLAND, J. L., STÖRK, C. K., SAUNDERS, J. H., HAMAIDE, A. J., BALLIGAND, M. H. & CLERCX, C. M. 2002. Intranasal infusion of enilconazole for treatment of sinonasal aspergillosis in dogs. *Journal of the American Veterinary Medical Association,* 221**,** 1421-1425.

Chapter 3. Australian environmental investigation for cryptic and pathogenic *Aspergillus* species and discovery of a novel species, *Aspergillus frankstonensis*

3.1 Introduction

This first study investigates how cats are exposed to the most common agent of feline upper respiratory tract aspergillosis *Aspergillus felis* in the Australian environment. Prior to this study, the majority of *A. felis* isolates had originated from clinical patients (5 human and 18 veterinary (1 dog, 17 cats)) (Barrs et al., 2013), with only a small number of environmental isolates located (one from an indoor air sample, four from soil in the USA, one each from soil in India, Zambia and the Czech Republic, one from a pine tree in Sri Lanka and one from food in Japan) (Barrs et al., 2013, Nováková et al., 2014). Interestingly, although the majority of clinical isolates of *A. felis* had originated in Australia (17/18) (Barrs et al., 2013), no isolates had yet been discovered in the Australian environment. Therefore an Australian environmental investigation was considered an important step in better understanding the pathogenesis of this disease and how cats are potentially exposed to infection by cryptic *Aspergillus* species. Additionally, given the ability of this fungus to cause disease in humans as well as animals, the prevalence of this species within the Australian environment was of relevance to both the veterinary and medical communities.

3.2 Premise

The publication in this chapter includes research into the environmental niche of *A. felis* and other opportunistic pathogenic cryptic species in the *A. viridinutans* species complex and *A.* section *Fumigati* in Australia.

This study utilised fungal environmental isolation techniques, including soil and air sampling. These substrates were selected as the most likely sources of *A. felis* based on previous environmental isolates (Barrs et al., 2013, Nováková et al., 2014). Agar type (dichloran rose-bengal agar with chloramphenicol (DRBC) and malt extract agar supplemented with chloramphenicol (MEASC), soil processing and incubation temperatures were chosen to favour growth of *Aspergillus viridinutans* species complex (AVSC) members, with the

inclusion of a heating diluted soil step to activate ascospores potentially in the soil (Houbraken and Samson, 2006, Kidd et al., 2009, Samson et al., 2010, Nováková et al., 2014). Morphological identification methods were then used to select *Aspergillus* species and molecular identification methods were used to identify genus and species level via amplification of the ITS region and partial beta-tubulin gene (White et al., 1990, Glass and Donaldson, 1995). Following this, other substrates were also tested, including cat food (dry commercial food) and different cat litter substrates.

This investigation spanning 2014 to 2016 included sampling locations along the east coast of Australia. For sample collection I visited the homes of cats that had been infected with aspergillosis, the majority of whom had succumbed to the disease. I developed a survey for the owner to complete regarding their cat's habits and husbandry related items (see Appendix 1). On arrival at their homes I discussed with owners their cat's case, habits and own environmental niches within their properties to attempt to narrow down collection sites.

During the investigation I recovered *Aspergillus* isolates whose species identity could not be resolved based on ITS and partial beta-tubulin sequencing due to low percentage homology on BLAST analysis of the sequences and further phylogenetic analysis of the isolates was required. I performed this at the Westerdijk Institute in the Netherlands, funded by an Endeavour Research Fellowship from the Australian Government under the supervision of Dr Jos Houbraken and Dr Robert Samson. Based on morphological analysis (measurements and description of growth on multiple agar types at different temperatures), further phylogeny evaluation (amplification of partial calmodulin, RPB2 and actin genes and the MCM7 gene using novel primers, with Mr Bayes and RAxML phylogenetic analysis in concordance with the genealogical concordance phylogenetic species recognition rules) and exometabolite analysis under the supervision of Dr Jens Frisvad at the Technical University of Denmark, I further characterise a suspected novel species as well as other AVSC isolates I had sampled from the Australian environment (Smedsgaard, 1997, Hong et al., 2005, Stamatakis, 2006, Nielsen et al., 2011, Ronquist et al., 2011, Sugui et al., 2014). At the Westerdijk Institute I had the added opportunity to examine AVSC clinical and environmental isolates within their Mycobank collection and was able to perform further sequencing of these isolates to include in the phylogenetic analysis when assessing the potential new species.

A bank of important isolates from this investigation have been deposited into the Mycobank private collection of Professor Vanessa Barrs at the University of Sydney as well as to the Westerdijk Institute public collection and Technical University of Denmark collection.

This paper represents the discovery of not only the prevalence of *Aspergillus* species in Australian domestic dwellings, but also the discovery of a novel species belonging to the AVSC in Australia, *A. frankstonensis* sp. nov. .

3.3 Publication

The following publication has been re-formatted here to suit the style of this thesis. The publication's in-text references remain in the style required by the journal (number bracket form) and the publication associated tables, figures, references and supplementary data follow the main text. This research is presented in its original publication form in **Appendix 2**. A list of all other references referred to in this chapter outside of section **3.3 Publication** can be found at the chapter's end (see **3.5 References**).

Talbot JJ, Houbraken J, Frisvad JC, Samson RA, Kidd SE, Pitt J, Lindsay S, Beatty JA, Barrs VR. Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for animal and human fungal pathogens. *PLoS ONE*. 2017; 12(8): e0181660. https://doi.org/10.1371/journal.pone.0181660.

Abstract

Invasive fungal infections (IFI) due to species in *Aspergillus* section *Fumigati* (ASF), including the *Aspergillus viridinutans* species complex (AVSC), are increasingly reported in humans and cats. The risk of exposure to these medically important fungi in Australia is unknown. Air and soil was sampled from the domiciles of pet cats diagnosed with these IFI and from a nature reserve in Frankston, Victoria, where *Aspergillus viridinutans sensu stricto* was discovered in 1954. Of 104 ASF species isolated, 61% were *A. fumigatus sensu stricto*, 9% were AVSC (*A. felis*-clade and *A. frankstonensis* sp. nov.) and 30% were other species (30%). Seven pathogenic ASF species known to cause disease in humans and animals (*A. felis*-clade, *A. fischeri*, *A. thermomutatus*, *A. lentulus*, *A. laciniosus A. fumisynnematus*, *A. hiratsukae*) comprised 25% of isolates overall. AVSC species were only isolated from Frankston soil where they were

abundant, suggesting a particular ecological niche. Phylogenetic, morphological and metabolomic analyses of these isolates identified a new species, *A. frankstonensis* that is phylogenetically distinct from other AVSC species, heterothallic and produces a unique array of extrolites, including the UV spectrum characterized compounds DOLD, RAIMO and CALBO. Shared morphological and physiological characteristics with other AVSC species include slow sporulation, optimal growth at 37˚C, no growth at 50˚C, and viriditoxin production. Overall, the risk of environmental exposure to pathogenic species in ASF in Australia appears to be high, but there was no evidence of direct environmental exposure to AVSC species in areas where humans and cats cohabitate.

Introduction

Aspergillosis is an opportunistic respiratory or systemic disease affecting a range of mammalian, avian and reptile hosts globally. It is most commonly caused by fungi belonging to *Aspergillus* section *Fumigati* (ASF), of which 19 of 63 described species are known to be pathogenic [1, 2]. The saprophytic, ubiquitous fungus *Aspergillus fumigatus sensu stricto* is the most common cause of aspergillosis overall. However, other species within the section, including members of the *Aspergillus viridinutans* species complex (AVSC) are increasingly recognized as emerging causes of invasive fungal infections (IFI). Currently, of the 10 accepted species in the AVSC, six are known pathogens including *A. udagawae*, *A. felis*, *A. wyomingensis*, *A. pseudoviridinutans*, *A. parafelis and A. pseudofelis* [2–8].

Aspergillus felis, *A. wyomingensis* and *A. udagawae* cause an invasive form of fungal rhinosinusitis in systemically immunocompetent cats called sino-orbital aspergillosis [2, 4]. Infection is often fatal, with high MICs of azole antifungals recorded *in vitro* and clinical resistance *in vivo* [4, 9]. *Aspergillus felis* has been isolated from clinical samples in humans, cats, dogs and birds, demonstrating a broad range of susceptible hosts [4]. While feline sino-orbital aspergillosis has been reported in diverse geographic regions including Europe, the United States and Japan, the majority of cases are from Australia [4, 10]. Other, less common causes of feline fungal rhinosinusitis include *A. fumigatus* s. str., *A. thermomutatus* (syn. *N. pseudofischeri*), *A. fischeri, A. lentulus* and *A. niger* species complex [9–13].

Aspergillus felis was first isolated from infected cats in Australia [4]. It has since been isolated from environmental samples from the USA and from other countries with low to no disease

incidence, in soil (Czech Republic, India, Zambia) [14], Caribbean Pine (Sri Lanka) [14], indoor air (Germany) [4] and on desert rocks in Chile [15]. The environmental prevalence of *A. felis* and other pathogenic species in section *Fumigati* in Australia has not been investigated, so environmental risk factors for exposure are unknown.

Here we report the findings of an investigation to determine if ASF species causing IFI in cats could be isolated from the home environments of cats with these infections. We also collected environmental samples from the region where *A. viridinutans* s. str. was first recovered and discovered a novel species, *Aspergillus frankstonensis* sp. nov.

Materials and methods

Sampling and isolation

With the permission of private land owners, as per the University of Sydney Human Ethics Approval (project number 2014/980), soil and air samples were collected from yards and gardens where eight cats previously diagnosed with SOA due to *A. udagawae* (n = 2), *A. felis* (n = 5) and *A. wyomingensis* (n = 1) were domiciled (5 in New South Wales (NSW), 2 in Victoria (VIC), 1 in the Australian Capital Territory (ACT). These locations included urban (n = 6), rural (n = 1) and semi-rural (n = 1) properties. Samples were also collected from municipal nature reserves in the coastal town of Frankston in the Mornington Peninsula region, VIC, where *A. viridinutans* s. str. was originally isolated in soil and rabbit dung [16]. Permission to sample was granted by the Frankston City Council. Field studies did not involve endangered or protected species.

A minimum of two air samples were collected at each site directly onto dichloran rose-bengal agar (DRBC) (Thermo Scientific, Thebarton, Australia) using a Merck Millipore MAS-100 NT1 air sampler (Merck KGaA, Darmstadt, Germany) at a rate of 100L/minute [17]. Ten soil samples, including garden and lawn soils (sandy and loamy) and mulches (sugar cane, wood chip and straw hay) were collected at each site to a depth of 10 cm and stored at 4˚C. Isolation of fungi from soil was achieved by 10−1 to 10−4 serial dilutions with sterile water. The resulting dilutions were transferred to a Whirlpak1 (Nasco) homogenizer bag, heated at 75˚C for 30 min, and 0.1 mL was inoculated onto DRBC [14, 18]. Additionally, 17 representative

samples of different soil types from each location were processed using the same method without heat treatment.

Samples of commercial cat litter substrates, including the same brands used by cats in the study (recycled paper pellets, clumping clay, recycled timber pellets and silica crystals), commercially available dry cat food samples were also tested in the same manner.

Air samples were incubated in the dark for up to three weeks at 37˚C. Treated soil samples were cultured on DRBC and malt extract agar supplemented with chloramphenicol (MEASC) [19] for up to three weeks at 37˚C. Fungal colonies were sub-cultured onto malt extract agar (MEA) (Thermo Scientific Oxoid Microbiology Products, Thebarton, Australia; Landsmeer, Netherlands) [19] for further analysis.

Identification of environmental isolates

For environmental isolates with gross macro- and microscopic morphological features consistent with *Aspergillus* spp. (excluding *Aspergillus* section *Nigri* species), amplification and sequencing of the ITS region and partial β-tubulin (*BenA*) gene was performed for species identification as previously described [20, 21]. A BLAST [22] search on GenBank was performed with the newly generated sequences, which were also aligned with *Aspergillus* references sequences (Samson et al. 2014) using MEGA version 6 software [23]. Phylogenetic analysis was performed using the maximum likelihood discrete method (tree searching method of 1000 replicate trees) and bootstrapping to determine the statistical support of the nodes.

Phylogenetic analysis of AVSC isolates. Nine environmental isolates identified in the AVSC were further examined based on molecular studies and phylogenetic analysis results of less than 100% match for *A. felis* (n = 7) and *A. viridinutans* (n = 2) ITS and *BenA* sequences. Additional molecular analysis was performed by sequencing partial calmodulin (*CaM*), actin (*Act*) and RNA polymerase II second largest subunit (*RPB2*) genes as previously described [8, 24]. Specific primers targeting the minichromosome maintenance factor gene (*Mcm7*) were developed based on previously published primers [8]: MCM7-709F_Fum ACTCGTGTCTCG GACGTCAAACC (forward) and MCM7-1348R_Fum GATTTGGCRACACCAGGATCACCCAT (reverse). For comparative analysis, these genes were also sequenced for AVSC members in the CBS-KNAW collection and for a new clinical

isolate from the USA from a cat with SOA (see Table 1). Phylogenetic and molecular evolutionary analyses were conducted using Randomised Axelerated Maximum Likelihood (RAxML) and Bayesian methods [25, 26].

Phenotypic species differentiation. The physiology and macro- and micromorphology of the two isolates demonstrated to be phylogenetically distinct from other AVSC species (CBS 142234 and CBS 142233) were studied. Isolates were grown at 25˚C on Czapek yeast agar (CYA) [19], Czapek yeast agar with 5% NaCl (CYAS) [27], yeast extract sucrose agar (YES) [19], MEA, oatmeal agar (OAT), creatine sucrose agar (CREA) [19], dichloran 18% glycerol agar (DG18) [28] for seven days. For temperature growth testing isolates were also grown on CYA at 30˚C, 37˚C, 45˚C and 50˚C for seven days.

Extrolite analysis. Extrolite extraction was performed on the two phylogenetically distinct isolates after growth on CYA and YES agar at 25˚C and 37˚C for 7 days. Three agar plugs were extracted according to the agar plug extraction method of Smedsgaard [29]. Extracts were analysed using UHPLC-DAD (Dionex Ultramate 3000 UHPLC) and compounds were identified against an internal database of UV spectra and literature [30]. Extrolite standards were available as reported by Nielsen et al. [30].

Antifungal susceptibility testing. Antifungal susceptibility testing was performed on all AVSC environmental isolates, and the clinical isolate, using Sensititre YeastOne YO8 microdilution trays (Trek Diagnostic Systems, Thermo Fisher Scientific, Scoresby, Australia) to assess the minimum inhibitory concentration (MIC) values of posaconazole (POS), itraconazole (ITZ), voriconazole (VCZ), fluconazole (FLU), ketoconazole (KCZ), amphotericin B (AMB), and minimum effective concentration (MEC) of caspofungin (CSP) as previously described [31].

Mating type analysis. Mating type for all AVSC environmental isolates was determined by targeting the MAT1-1 and MAT1-2 genes [4]. Mating experiments were also performed on isolates with opposite mating types of the same species where available, or with other members of the AVSC where unavailable, on OAT and MEA in the dark at 30˚C. Ascospore viability tests were performed by rupturing ascomata, suspending ascospores in 0.05% Tween 80 and heating at 70˚C for 60 min. After heating, 100 μL of the ascospore suspension was plated on 2% MEA and incubated at 28˚C for 24 h [32]. To act as a negative control, the same treatment was also applied to the conidia of paired parental strains from the mating plate. Scanning

electron microscopy was performed on all ascospores (Emitech 550K Sputter coater JEOL 6480LA).

Nomenclature. The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: PubMed Central, LOCKSS.

Results

Sequence-based identification

Overall 104 ASF species were isolated from all sites including 61% (n = 64) *A. fumigatus s. str*., 9% (n = 9) AVSC (*A. felis*-clade and *A. frankstonensis* sp. nov.) and 30% (n = 31) other ASF species (see S2 Table, S1 Fig) (Genbank accession numbers: KY808753-KY808856 (ITS); KY808591- KY808694 (*BenA*)). Pathogenic ASF species (*A. A. felis*-clade (n = 7), *A. fischeri* (n = 6), *A. thermomutatus* (n = 1), *A. lentulus* (n = 2), *A. laciniosus* (n = 2), *A. fumisynnematus* (n = 4), *A. hiratsukae* (n = 1) comprised 25% of isolates overall. AVSC species were only isolated from Frankston soil where they were abundant and comprised 41% of isolates from that site.

Aspergillus isolates were only recovered from three of the nine sampled locations when no heat treatment was used. Isolated species included *A. fumigatus* s. str. (Kealba, n = 1), *A. hiratsukae* (Frankston, n = 1; Amaroo, n = 1). *Aspergillus fumigatus* s. str. was also isolated from recycled paper cat litter. No *Aspergillus* species were isolated from other cat litter types and dry food

tested. The clinical isolate from the cat in the US (Table 1) was phylogenetically closely related to the type of *A. parafelis*, CBS 140762, in the *A. felis*-clade.

Phylogeny. The length of the datasets were *BenA* 422 basepairs (bp), *CaM* 485 bp, *Act* 379 bp, *RPB2* 831 bp and *Mcm7* 450 bp. Phylogenetic analysis of combined *BenA, CaM, Act, RPB2* and *Mcm7* data (2567 bp) confirmed seven isolates belong in the *A. felis*-clade, related to the type of *A. parafelis* CBS 140762, and two isolates were most closely related to, but phylogenetically distinct from *A. viridinutans* s. str. (Figs 1 and 2). These two isolates had identical sequences and are described as a new species below, *A. frankstonensis* sp. nov.

The individual and combined phylogenies (*BenA, CaM, RPB2, Act, Mcm7*) show that the 9 isolates from Frankston soil are accommodated in the AVSC and ASF (Figs 1 and 2). Two of these isolates (CBS 142233, CBS 142234) were most closely related to *A. viridinutans* s. str. in all trees, with bootstrap support (bs) >70% in four of the six trees generated (*RPB2* 71%bs; *Act* 95% bs; *BenA* 74% bs; combined 100% bs). This was also supported by Bayesian method posterior probability values (*RPB2* 0.99 pp; *Act* 1.00 pp; *BenA* 0.97 pp; combined 1.00 pp). *Aspergillus arcoverdensis* takes a basal position to those two species with high statistical support (bs 100%, 1.00 pp). These sequences of CBS 142234 and CBS 142233 are different from the other species in AVSC and ASF, with the genetic change seen in the distance of the horizontal branch. For both these isolates the percentage difference at ITS, *BenA, CaM, RPB2, Act* and *Mcm7* were 99%, 96%, 98%, 99%, 98% and 98% respectively when compared with *A. viridinutans* s. str. (NRRL 4365). A BLAST analysis did not show a 100% similarity match on GenBank for either of these isolates, and the highest similarities were with the type strain for *A. viridinutans* s. str. The remaining seven Frankston soil isolates (DTO 341-F2, DTO 341-E6, DTO341-E4, DTO 341-E5, DTO 341-F1, DTO 341-E9, DTO 341-E8) were also shown to be accommodated in the AVSC and ASF. The results of the analysis of the combined dataset (Fig 1) showed that these isolates were with high statistical support (99% bs, 1.00 pp) most closely related to the type of *A. parafelis* CBS 140762. The individual and combined phylogenies also showed that isolate CBS 458.75 is accommodated in the AVSC, most closely related to *A. pseudoviridinutans* isolates in five of the six trees generated (*Act* 70% bs, 0.96 pp; *CaM* 96% bs, 1.00 pp; *Mcm7* 89% bs 1.00 pp; *RPB2* 91% bs, 0.98 pp; combined 100% bs, 1.00 pp). Isolates DTO 131-E4, DTO 131-E5 and DTO 131-G1 formed a separate clade to other *A. felis* isolates in the *Act* (98% bs, 1.00 pp), *Mcm7* (86% bs, <0.95 pp), *RPB2* (<70% bs, 0.96 pp) and combined trees (89%, 1.00 pp). The *BenA* tree showed DTO 131-G1 and DTO 131-E5 were in

the same clade (100% bs, 1.00 pp) and basal to the *A. felis*-clade, while DTO 131-E4 was more closely related to the type of *A. felis* (CBS 130245) (70% bs, 0.99 pp). In the *CaM* tree, DTO 131-E4 and DTO 131-G1 were in the same clade (100% bs, 1.00 pp) but their position in the *A. felis*-clade was unresolved, and DTO 131-E5 was positioned in a clade with moderate statistical support (73% bs, < 0.95 pp) related to the types for *A. pseudofelis* (CBS 140763) and *A. felis* (CBS 130245).

Taxonomy

Morphological and physiological characterization

Species description of *A. frankstonensis* (CBS 142233 = DTO 341-E7 = IBT 34172; CBS 142234 = DTO 341-F3 = IBT 34204)

Aspergillus frankstonensis Talbot et al 2017, sp. nov. MycoBank 819986. Fig 3.

Etymology. Named after Frankston, Australia, the collection location of the type strain. This town in Victoria, Australia was also the location where *Aspergillus viridinutans s. str*. was first isolated [16].

Diagnostic characteristics. *Aspergillus frankstonensis* belongs in *Aspergillus* subgenus *Fumigati* section *Fumigati* and is phenotypically similar to other members of the AVSC as it is generally slow to sporulate and thermophilic. The species is phylogenetically most closely related to *A. viridinutans s. str*. but differs from this species by its ability to sporulate well at 37˚C and grow at 45˚C.

Specimen examined. Australia (latitude 38.1414˚S, longitude 145.1225˚E), from soil, collection date May 7th 2015, J. Talbot & V. Barrs, (holotype CBS-H-22969, culture ex-type CBS 142233 = DTO 341-E7; Australia, identical collection information as CBS 142233, CBS 142234 = DTO 341-F3).

Description (Fig 3)

Colony diam, 7 days, 25˚C (mm): CYA 30–43; MEA 28–34; YES 42–55; DG18 40–45; CYAS 18–22; OAT 40–43; DG18 20–24; CREA 10–27, poor growth, no acid production. Other incubation temperatures: CYA 30˚C 42–53; CYA 37˚C 45–56; CYA 45˚C 25; CYA 50˚C no growth; optimum growth temperature 37˚C, maximum between 45 and 50˚C.

Macromorphology: CYA 25˚C, 7 days: colony sulcate, radiating and concentric patterns; sporulation moderate; colony texture velvety to floccose; conidia yellow-green in centre of colony, dull green towards edge; non-sporulating edge 2 mm; mycelium white to yellow; soluble pigment present, yellow; exudate present as yellow droplets; margin regular; reverse yellow.

MEA 25˚C, 7 days: colony slightly raised, sulcate, radiating; sporulation poor; colony texture velvety to floccose; mycelium white to pale yellow; conidia pale to bright green; exudate present as clear to yellow droplets; reverse yellow to orange. YES 25˚C, 7 days: colony sulcate; sporulation moderate; mycelium white; conidia *en masse* pale-dull green; non-sporulating edge 3 mm; soluble pigments orange; exudate absent; reverse brown and yellow. DG18 25˚C, 7 days: colony sulcate; sporulation moderate, conidia dull green, mycelium white with pink tinge; soluble pigment present, pink to yellow; exudate absent, reverse yellow to brown. OA 25˚C, 7 days: colony elevated; sporulation moderate; colony texture floccose; mycelium white; conidia pale green; soluble pigment absent; exudate absent; sclerotia absent; reverse yellow. Micromorphology: Conidial heads columnar, uniseriate. Stipes hyaline, smooth walled,

60–130 × 4–5 µm. Vesicles subglobose, up to 12.5 µm in diameter. Phialides ampulliform, 6–8 × 2–3 µm, covering *75% of the head. Conidia globose, smooth, hyaline 2 × 2–3 µm; average width/length = 0.95, n = 40; Hülle cells absent.

Occurrence. This species has been found in the soil at a recreational reserve, Upper Sweetwater Creek Reserve in Frankston, Victoria, Australia, 38.1580˚ S, 145.1350˚ E.

Genbank accession numbers: CBS 142233: KY808756 (ITS); KY808594 (*BenA*); KY808724 (*CaM*); KY808948 (*RPB2*); KY808549 (*Act*); KY808901 (*Mcm7*). CBS 142234: KY808761(ITS);

KY808599 (*BenA*); KY808729 (*CaM*); KY808953 (*RPB2*); KY808554 (*Act*); KY808906 (*Mcm7*).

Secondary metabolite production

Both isolates produced viriditoxin, apolar indole-alkaloids and three new compounds given the temporary names of DOLD, RAIMO and CALBO, based on their unique UV spectra. The CALBO compounds had an absorption maximum at 343 nm quite similar to calbistrins. One of the isolates (CBS 142234) was also observed to produce two chrysogine precursors, a unique apolar indol alkaloid and an additional three new compounds given the temporary names of OKAM, USOC and COT based on their UV spectra. The other isolate (CBS 142233) produced aszonapyrone A & B, a chrysogine precursor, apolar indolalkaloids and an additional two compounds given the temporary name of HITO and BRUDA. *Aspergillus frankstonensis* shares viriditoxin production with *A. viridinutans*, and aszonapyrones with several *Aspergillus* section *Fumigati* species.

Antifungal susceptibilities

Antifungal susceptibility results are summarized in Table 2 for two *A. frankstonensis* isolates and seven *A. felis*-clade isolates (6 environmental, 1 clinical). There was no observed activity of ITZ against one environmental *A. felis*-clade isolate.

Mating type analysis

Both isolates of *A. frankstonensis* sp. nov. were MAT1-2. All pairings with MAT1-2 isolates of other AVSC species were negative (supplementary Table 2). Both mating types MAT1-1 (n = 5) and MAT1-2 (n = 2) were found amongst *A. felis*-clade environmental isolates. Positive intra-species and inter-species matings between opposite mating types (Table 3) produced clusters of white to creamish cleistothecia along the barrage zone that contained lenticular ascospores with two prominent equatorial crests and an echinulate convex surface. Ascospores from three *A. felis*-clade intra-species and one inter- species pairing with *A. felis* were fertile and from one pairing with *A. wyomingensis* were infertile (Fig 4). No growth was seen from parental strains.

Taxonomic notes

Aspergillus frankstonensis has some unique morphological characteristics that can be used to further distinguish it from its closest AVSC relatives (including *A. viridinutans s. str*., *A. arcoverdensis* and *A. udagawae*). Grossly, when grown on CYA at 25˚C in the dark for 7 days, *A. frankstonensis* has a smaller colony diameter than *A. arcoverdensis* and *A. udagawae* (*A. frankstonensis* 30–43 mm; *A. arcoverdensis* 56–58 mm; *A. udagawae* 82–85 mm), and a larger colony diameter than *A. viridinutans* (28–40 mm) [14, 33]. On MEA *A. frankstonensis* has pale to bright green conidia on white to pale yellow mycelium, whereas its closely related species range from yellowish white (*A. arcoverdensis*) to gray green (*A. viridinutans*) to dull green (*A. udagawae*) [33]. Microscopically, the conidial head of *A. frankstonensis* (60– 130 × 4–5 μm) differs in size compared to *A. arcoverdensis* (82–110 × 22.5–30 μm), *A. viridinutans* (50 × 30 μm) and *A. udagawae* (95–145 × 20–50 μm) [33]. It differs from *A. fumigatus* by its inability to grow at 50˚C.

Discussion

Our findings suggest that human and animal exposure to pathogenic *Aspergillus* species in Australia is not uncommon since 25% of all ASF isolates were pathogenic species. As expected, the predominant *Aspergillus* species isolated was *A. fumigatus s. str*. and this species remains the most common cause of aspergillosis in humans and animals. However, the incidence of aspergillosis due to other species in ASF is increasing, reported between 3 to 5% of aspergillosis cases in human patients [34–38].

In soil from residential environments we found the human and animal pathogens *A. fumigatus, A. fischeri, A. laciniosus, A. lentulus, A. fumisynnematus, A. hiratsukae* and *A. thermomutatus* (syn. *N. pseudofischeri*). Four of these (*A. fumigatus, A. fischeri, A. lentulus* and *A. thermomutatus*) cause IFI in cats [4, 10]. Isolation of these known feline pathogens from soil in areas cats had access to supports that the source of feline infections is environmental. *A. felis* conidia have also been found in air [4], making infection of cats possible via disruption of soil or wind dispersal, such as could occur through the natural feline behaviours of digging, sniffing and grooming. Given that infection often occurs in brachycephalic purebred cats of Persian lineage [4, 9], an immunogenetic defect may predispose cats to disease [10, 39].

Interestingly, we only isolated AVSC species from soil from Frankston, but not from residential environments, cat litter or cat food. *A. viridinutans s. str*., was discovered in Frankston in 1954 in rabbit dung and sandy soil [16]. This suggests a possible ecological niche for AVSC species at this site associated with faunae, flora and associated soils [40], or local processes such as bush regeneration and back-burning which may remove competing microbiota [41, 42]. AVSC species have been found to be abundant in other specific regions, including a coal mining reclamation site in Wyoming, USA [14].

The most abundant AVSC species isolated from Frankston were in the *A-felis*-clade, closely related to the type of *A. parafelis*, CBS 140762, which is a clinical isolate from the oropharyngeal exudate of a human [8]. Here, we also identified another closely related isolate, OHIGB6-A1, which was cultured from a cat with sino-orbital aspergillosis.

Our soil isolation technique was adapted from two previous studies [14, 18]. As *A. felis* is a heterothallic species producing heat resistant ascospores, we aimed to recover activated ascospores by heat treating soil. Soil processed without heat did not recover any members of the AVSC. Thus, the ascospores of these species are present in soil and heat activation appears to be an important surveillance technique for heterothallic AVSC species.

Following their polyphasic taxonomical analysis of 11 AVSC isolates, Sugui et al (2014) described three phylogenetically closely related but separate species within the *A. felis*-clade; the already described *A. felis* [4] and two new species, *A. parafelis* and *A. pseudofelis*. We investigated the phylogenetic relationships of 56 clinical and environmental AVSC isolates (Fig 1). Our phylogenetic data, based on the single gene and a combined dataset of six genes demonstrated that *A. felis* and *A. pseudofelis* are the same species. The distinctiveness of *A. parafelis* is questionable due to the positioning of a clade of previously described *A. felis* isolates DTO 131 E4, DTO 131-E5 and DTO 131-G1 [4]. These isolates have in the combined dataset a basal position to the *A. felis*-clade, but the position of these isolates in the single gene phylogenies isn't congruent. This data indicates that *A. parafelis* is also conspecific with *A. felis*, when the gene concordance phylogenetic species recognition concept is applied. This is supported by mating experiments and isolates from different lineages are all able to mate [8]. We demonstrated fertile mating between *A. felis* (DTO 131-F4) and an *A. felis*-clade isolate (DTO 341-E6), which is closely related to the type strain for *A. parafelis* (CBS 140762).

Our phylogenetic data also showed that isolate CBS 458.75 is phylogenetically most closely related to *A. pseudoviridinutans*. Investigation into exometabolite production will further determine the relationship, however preliminary studies have shown CBS 458.75 is able to produce some of the same exometabolites that *A. pseudoviridinutans* strains (DTO 303-A1, NRRL 6106) produce, including antafumicins, clavatols, fumigatin, VERN and pseurotins.

Here we reported the discovery of a new AVSC species, *A. frankstonensis*, which is of unknown pathogenicity. However, the ability to sporulate at 37˚C indicate pathogenic potential [1]. Although MICs of most antifungals tested were generally low, one *A. frankstonensis* isolate had a high MIC of VCZ (4 ug/mL).

We performed AVSC inter-species mating experiments on *A. frankstonensis* sp. nov. as other AVSC members have been reported to mate with other species in the complex [8]. *A. frankstonensis* inter-species mating were negative, further confirming its status as a distinct species. Intraspecies mating tests could not be performed as we had only one mating type for *A. frankstonensis*. Heterothallism (sexual reproduction) allows genetic recombination and has the potential to increase fitness. This may be beneficial for adaptation to environmental conditions, and may also contribute to drug resistance [43]. Interestingly, the majority of the 19 known pathogenic fungi from the genus *Aspergillus* are also heterothallic, with the exception of some doubtful species, *A. beijingensis*, *A. qizutongi* and *A. wanduanglii* [44]. However, many heterothallic species of unknown pathogenicity also exist.

Recent studies have demonstrated that the small molecule extrolite (secondary metabolite), profiles of ASF species can determine the relatedness and identification of a species [45], and may also predict the potential pathogenicity of a new species where only environmental isolates have been discovered [1]. Extrolite production by AVSC environmental isolates in this study shared similarities with other members in the complex. All isolates were shown to produce viriditoxin, which is produced by all other members of the AVSC and one other ASF species [1], therefore its link to pathogenicity is unknown. The only other ASF species reported to produce viriditoxin is *A. denticulatus* [46]. There were some secondary metabolite differences between the two *A. frankstonensis sp. nov*. isolates from Frankston soil. One isolate produced aszonapyrones and chrysogine precursors which may be associated with pathogenicity [47, 48]. Aszonapyrones have antibacterial properties [47]; and chrysogine is an alkaloid [48]. There was a notable difference in the degree of sporulation between the two isolates with one

demonstrating poor sporulation. This may account for the differences in extrolite profiles. However, phylogenetically they are the same species with no nucleotide differences between them, based on a number of targeted genes. Novel extrolites were also produced by these isolates, some of which were shared. These unique extrolites may be produced for fungal competitiveness in the primary habitat of this fungal species. Further analysis of the novel compounds and secondary metabolite profiling of other members of the AVSC will be undertaken by the authors for further comparison between environmental and pathogenic strains.

Conclusions

The risk of exposure to pathogenic species in ASF in Australia appears to be high. The risk of direct environmental exposure to the AVSC in areas where humans and cats co-habitate in Australia, however appears to be low. There was no evidence of an environmental reservoir of these organisms in the homes of any cats diagnosed with aspergillosis. Detection of AVSC organisms from only one location suggests a niche for these species that favours specific environmental conditions. *A. frankstonensis* sp. nov is an interesting new species in ASF that is closely related to known human and animal pathogens and possesses some virulent characteristics including growth at 37˚C and a high MIC of voriconazole. It also produces unique secondary metabolites that require further investigation.

Acknowledgments

The authors wish to thank Martin Meijer and Tineke van Doorn at Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands for technical assistance.

Publication Figures

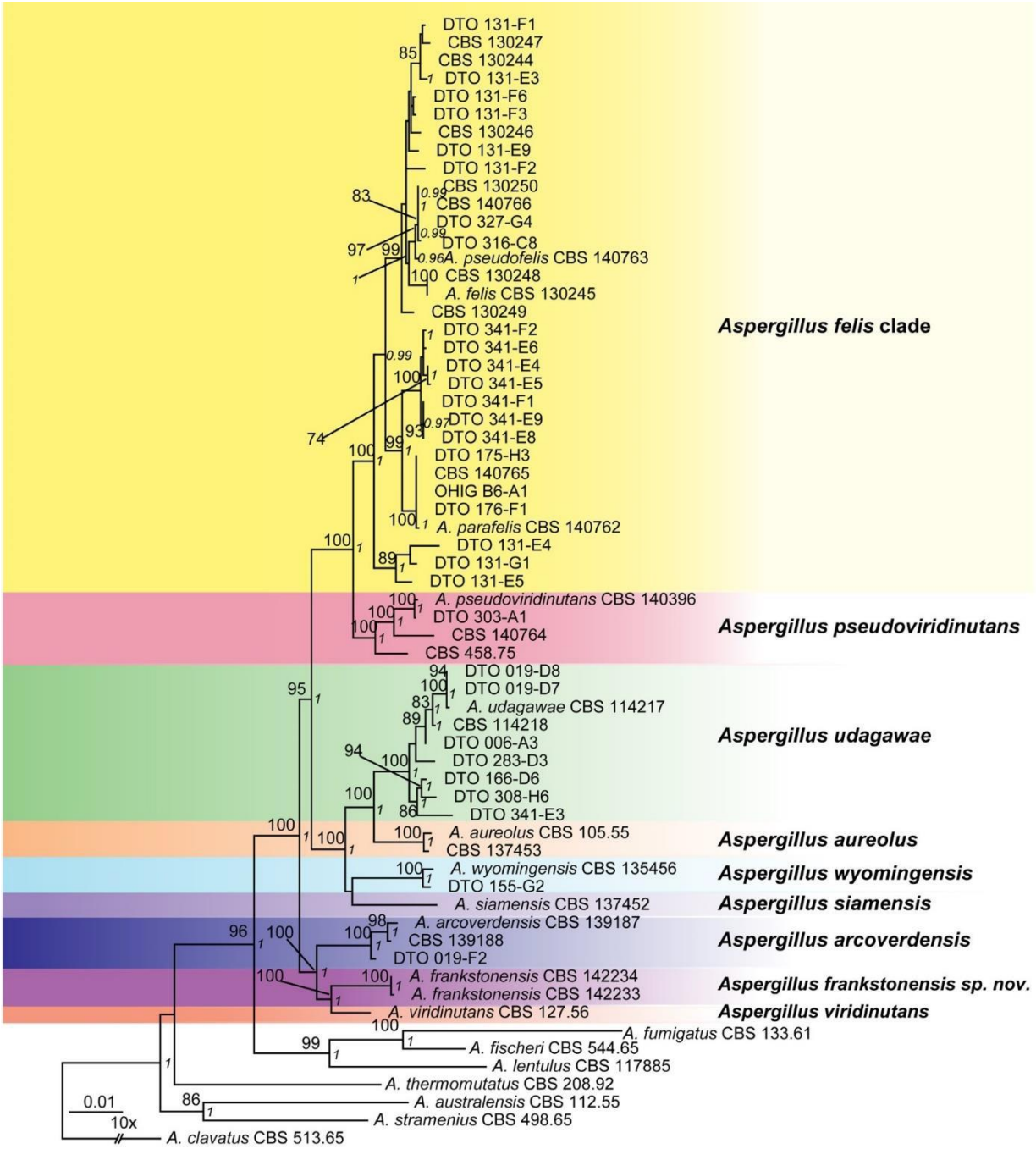


Figure 1. *Aspergillus viridinutans* species complex combined phylogenetic tree. Tree based on sequencing of *Mcm7, BenA, Act, RPB2, CaM* genes (phylogeny model Kimura 2, gamma distribution, 1000 bootstrap replicates with Bayesian method posterior probability values in italics). Isolates previously described as *A. felis, A. parafelis* and *A. pseudofelis* are listed here under the one grouping "*A. felis*-clade".

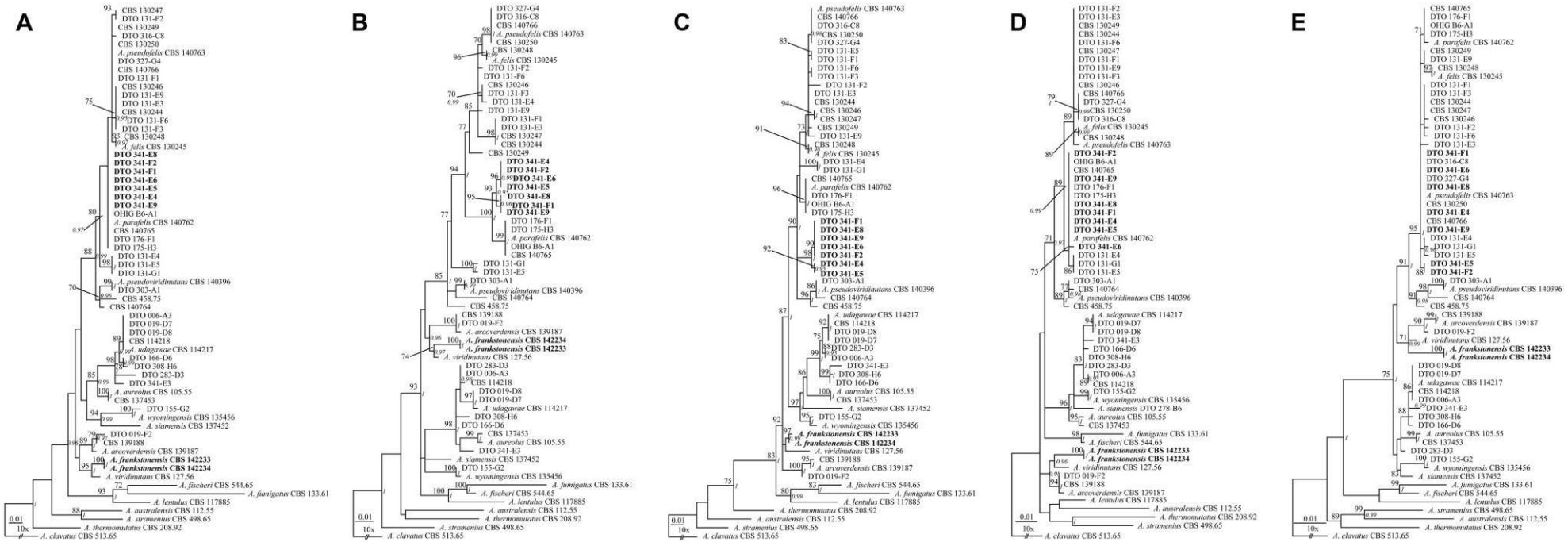


Figure 2. *Aspergillus viridinutans* species complex individual gene phylogenetic trees. All phylogenies made with gamma distribution and 1000 bootstrap replicates with Bayesian method posterior probability values in italics. A = *Act* (phylogeny model General Time Reversible); B = *BenA* (phylogeny model Kimura 2); C = *CaM* (phylogeny model Tamura-Nei); D = *Mcm7* (phylogeny model Kimura 2); E = RPB2 (phylogeny model General Time Reversible).

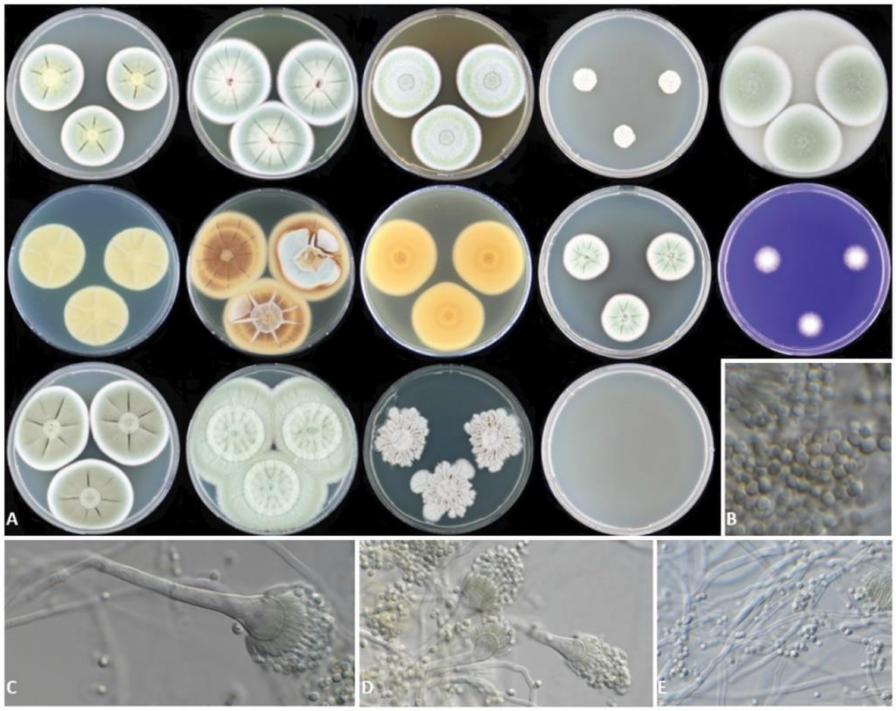


Figure 3. *Aspergillus frankstonensis* CBS 142233; CBS 142234. (A) Colonies grown at 25˚C for 7 days, from left to right (top row) CYA, YES, MEA, CYAS, OA; middle row CYA reverse, YES reverse, MEA reverse, DG18, CREA; bottom CYA grown at 30˚C, 37˚C, 45˚C, 50˚C. (B) Conidia (C—D) Conidiophores (E) Hyphae.

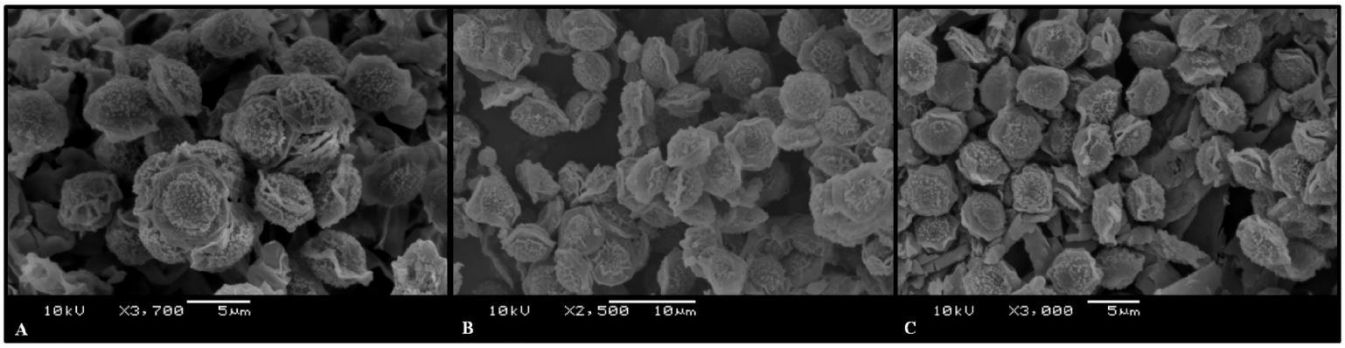


Figure 4. Ascospores from mating experiments with A. felis-clade isolates. A = A. felis-clade (DTO 341-E6) X A. felis-clade (DTO 341-E5); B = A. felis-clade (DTO 341-E6) X A. wyomingensis (CCF 4416; C = A. felis-clade (DTO 341-F2) X A. felis (DTO 131-E3).

Table 1. Isolates included in phylogenetic analysis of the *Aspergillus viridinutans* complex.

Identification number	**Species name**	**Source**	**Location**	**ITS**	**BenA**	**Cam**	**RPB2**	**Actin**	**MCM7**
DTO 006-A3	*A. udagawae*	soil	USA	KY808735	KY808572	KY808696	KY808908	KY808509	KY808858
DTO 019-D7	*A. udagawae*	unknown	unknown	KY808737	KY808573	KY808697	KY808909	KY808510	KY808859
DTO 019-D8	*A. udagawae*	unknown	unknown	KY808738	KY808574	KY808698	KY808910	KY808511	KY808860
DTO 019-F2	*A. arcoverdensis*	soil	Australia	KY808747	KY808575	KY808699	KY808911	KY808512	KY808861
DTO 050-F1, CBS 127.56^{T}, NRRL 4365	*A. viridinutans*	rabbit dung	Australia	EF669978	AF134779	DQ534162	EF669765	DQ094862	KY808862
DTO 331-G6, CBS 105.55^{T}, DTO 052-C8, NRRL 2244	*A. aureolus*	soil	Ghana	EF669950	EF669808	KY808720	KY808943	DQ094861	KY808895
DTO 131-E3	*A. felis*	cat, RBM	Australia	JX021671	KY808576	KY808701	KY808912	KY808513	KY808863
DTO 131-E4	*A. felis*	cat, RBM	Australia	JX021673	JX021692	KY808702	KY808913	KY808514	KY808864
DTO 131-E6, CBS 130244	*A. felis*	cat, RBM	Australia	JX021675	JX021694	JX021717	KY808915	KY808516	KY808866
DTO 131-E5	*A. felis*	cat, RBM	Australia	JX021674	JX021693	JX021719	KY808914	KY808515	KY808865
DTO 131-E9	*A. felis*	cat, RBM	Australia	JX021676	JX021696	KY808703	KY808916	KY808517	KY808867
DTO 131-F1	*A. felis*	cat, RBM	Australia	JX021677	JX021697	KY808704	KY808917	KY808518	KY808868
DTO 131-F2	*A. felis*	cat, RBM	Australia	JX021678	JX021698	KY808705	KY808918	KY808519	KY808869
DTO 131-F3	*A. felis*	cat, RBM	Australia	JX021679	JX021699	KY808706	KY808919	KY808520	KY808870
DTO 131-F4, CBS 130245^{T}	*A. felis*	cat, RBM	Australia	J X021685	JX021700	JX021715	KY808920	KY808521	KY808871
DTO 131-F6	*A. felis*	cat, RBM	Australia	JX021680	JX021702	JX021721	KY808921	KY808522	KY808872
DTO 131-F9, CBS 130246	*A. felis*	cat, SNC	Australia	JX021681	JX021704	JX021724	KY808922	KY808523	KY808873
DTO 131-G1	*A. felis*	cat, RBM	Australia	JX021682	JX021705	JX021725	KY808923	KY808524	KY808874
DTO 131-G2, CBS 130247	*A. felis*	cat, RBM	Australia	JX021683	JX021706	JX021726	KY808924	KY808525	KY808875

DTO 131-G3, CBS 130248	*A. felis*	cat, RBM	Australia	JX021684	JX021707	JX021727	KY808925	KY808526	KY808876
DTO 155-G2	*A. wyomingensis*	cat, RBM	Australia	JX021685	JX021709	KY808707	KY808927	KY808527	KY808878
DTO 155-G3, CBS 130249	*A. felis*	dog, VH	Australia	JX021686	JX021711	JX021713	KY808928	KY808528	KY808879
DTO 157-D7, CBS 114217^{T}	*A. udagawae*	soil	Brazil	AB250781	AF132226	AB748566	KY808929	KY808529	KY808880
DTO 157-D8, CBS 114218	*A. udagawae*	soil	Brazil	AB250782	AB248303	AY689373	KY808930	KY808530	KY808881
DTO 159-C9, CBS 130250	*A. felis*-clade	cat, RBM	United Kingdom	JX021689	JX021712	JX021714	KY808931	KY808531	KY808882
DTO 166-D6	*A. udagawae*	cat	Australia	KY808740	KY808579	KY808710	KY808932	KY808532	KY808883
DTO 175-H3	*A. sp.*	surface water	Portugal	KY808741	KY808580	KY808711	KY808933	KY808533	KY808884
DTO 176-F1	*A. felis*	air	Germany	KY808742	KY808581	KC305168	KY808934	KY808534	KY808885
DTO 278-B6, CBS 137452^{T}	*A. siamensis*	soil	Thailand	-	KY808582	AB776704	KY808712	KY829134	KY808886
DTO 278-B7, CBS 137453	*A. aureolus*	soil	Brazil	KY808743	KY808583	KY808713	KY808935	KY808535	KY808887
DTO 283-D3	*A. udagawae*	soil	Thailand	KY808744	KY808584	KY808714	KY808936	KY808536	KY808888
DTO 303-A1	*A. pseudoviridinutans*	*Pinus caribea* (pine tree)	Sri Lanka	KY808745	KY808585	KY808715	KY808937	KY808537	KY808889
DTO 308-H6	*A. udagawae*	soil	Turkey	KY808746	KY808586	KY808716	KY808938	KY808539	KY808890
DTO 316-C8	*A. felis/ A. conversis*	CBS culture contaminant	The Netherlands	KY808750	KY808587	KY808717	KY808939	KY808540	KY808891
DTO 316-F7, CBS 139187^{T}	*A. arcoverdensis*	semi-desert soil	Brazil	KY808748	AB818845	AB818856	KY808940	KY808541	KY808892
DTO 316-F9, CBS 139188	*A. arcoverdensis*	unknown	Brazil	KY808749	KY808588	KY808718	KY808941	KY808542	KY808893
DTO 327-G4	*A. viridinutans*	human patient	The Netherlands	KY808751	KX903288	KY808719	KY808942	KY808543	KY808894
DTO 332-B1, CBS 135456^{T}	*A. wyomingensis*	coal mine reclamation site soil	Glenrock, USA	HG324081	KY808589	KY808721	HF937378	KY808544	KY808896

NRRL 6106, DTO 342-I3, CBS 140764	*A. pseudoviridinutans*	unknown	Unknown	-	AF134778	KJ914709	KY808965	KY808566	KJ914726
NRRL 62900, CM-3147, DTO 342-I4, CBS 140762[T]	*A. felis*-clade	human, OPE	Spain	-	KJ914692	KJ914702	KY808966	KY808567	KJ914720
NRRL 62901, CM-5623, DTO 342-I5, CBS 140765	*A. felis*-clade	human, lungs	Portugal	-	KJ914693	KJ914703	KY808967	KY808568	KJ914721
NRRL 62902, CM-4518, DTO 342-I6, CBS 140766	*A. felis*-clade	human, nail	Spain	-	KJ914696	KJ914704	KY808968	KY808569	KJ914722
NRRL 62903, CM-6087, DTO 342-I7, CBS 140763[T]	*A. felis*-clade	human, sputum	Spain	-	KJ914697	KJ914705	KY808969	KY808570	KJ914723
NRRL 62904, NIHAV1, DTO 304-I5, CBS 140396[T]	*A. pseudoviridinutans*	human, lung	USA	-	GQ144441	GQ144442	KJ914730	KY808538	KJ914727
DTO 341-E8	*A. felis*-clade	woodland soil	Frankston, Australia	KY808757	KY808595	KY808725	KY808949	KY808550	KY808902
DTO 341-E9	*A. felis*-clade	woodland soil	Frankston, Australia	KY808758	KY808596	KY808726	KY808950	KY808551	KY808903
DTO 341-F1	*A. felis*-clade	woodland soil	Frankston, Australia	KY808759	KY808597	KY808727	KY808951	KY808552	KY808904

DTO 341-E6	*A. felis*-clade	woodland soil	Frankston, Australia	KY808755	KY808593	KY829133	KY808947	KY808548	KY808900
DTO341-E4	*A. felis*-clade	woodland soil	Frankston, Australia	KY808753	KY808591	KY808723	KY808945	KY808546	KY808898
DTO 341-F2	*A. felis*-clade	woodland soil	Frankston, Australia	KY808760	KY808598	KY808728	KY808952	KY808553	KY808905
DTO 341-E5	*A. felis*-clade	woodland soil	Frankston, Australia	KY808754	KY808592	KY829132	KY808946	KY808547	KY808899
OHIG B6-A1	*A. felis*-clade	cat, RBM	Connecticut, USA	KY808857	KY808695	KY808734	KY808970	KY808571	KY808907
DTO 341-E7, CBS 142233, IBT 34172 [T]	*A. frankstonensis* sp. nov.	woodland soil	Frankston, Australia	KY808756	KY808594	KY808724	KY808948	KY808549	KY808901
DTO 341-F3, CBS 142234, IBT 34204	*A. frankstonensis* sp. nov.	woodland soil	Frankston, Australia	KY808761	KY808599	KY808729	KY808953	KY808554	KY808906
DTO 341-E3, CBS 142231	*A. udagawae*	cat, RBM	Kealba, Australia	KY808752	KY808590	KY808722	KY808944	KY808545	KY808897
DTO 153-A1*, CBS 458.75	*A.* sp.	soil	India	KY808736	AY685178	HG426048	KY808926	DQ094853	KY808877

[T] = Type strain; CBS ID number culture collection of the Westerdijk Fungal Biodiversity Institute, the Netherlands; DTO in-house collection ID number at Westerdijk Institute, the Netherlands; NRRL ID number Agricultural Research Service Culture Collection, USA; OHIG ID number One Health Infectious Disease Research Group Collection, University of Sydney, Australia; RBM = retrobulbar mass; SNC = sino-nasal cavity; VH = vitreous humor; OPE = oropharyngeal exudates. * = This isolate was included in this study as previous in-house sequence analysis showed close phylogenetic relatedness to members of this complex. - = no sequence or accession number available.

Table 2. Antifungal susceptibility results for two *A. frankstonensis* isolates, six *A. felis*-clade isolates from environmental soil and one *A. felis*-clade clinical isolate (cat). MIC/MEC (µg/mL) values reflect the number of isolates within the specific cut-off value.

Drug	**Species**	**MIC/MEC (µg/mL) distribution among tested isolates**												
		0.015	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>16**	**GM**
AMB*	*A. frankstonensis*				1			1						0.35
	A. felis-clade							1	5	1				2.00
ITZ*	*A. frankstonensis*				1		1							0.24
	A. felis-clade							4	2				1	1.26
VCZ*	*A. frankstonensis*							1		1				2.00
	A. felis-clade							1	2	4				2.69
POS*	*A. frankstonensis*					1	1							0.35
	A. felis-clade						1	6						0.90
CSP†	*A. frankstonensis*	1	1											0.02
	A. felis-clade	5	1	1										0.02

*MIC, minimum inhibitory concentration; †MEC, minimum effective concentration; GM, geometric mean

Table 3. Intra- and interspecific mating results among environmental *A. felis*-clade isolates.

	MAT1-1 strain						
	***A. felis*-clade**	***A. felis*-clade**	***A. felis*-clade**	***A. felis*-clade**	***A. felis*-clade**	***A. felis***	***A. felis***
MAT1-2 strain	**DTO 341-F1**	**DTO 341-E6**	**DTO 341-E4**	**DTO 341-E8**	**DTO 341-E9**	**DTO 131-E3**	**DTO 131-E5**
A. felis-clade **DTO 341-F2**	+	+*	-	-	+	+	+
***A. felis*-clade** **DTO 341-E5**	+*	+*	-	-	-	-	-
A. felis **DTO 131-E4**	-	+*	+	-	-	-	-
A. wyomingensis **CCF 4416**	-	+	-	-	-	-	-
A. frankstonensis **DTO 341-F3**	-	-	-	-	-	-	-

$^{-}$ no cleistothecia or ascospores; $^{+}$ cleistothecia production; *fertile ascospores

Publication References

1. Frisvad JC, Larsen TO. Extrolites of *Aspergillus fumigatus* and other pathogenic species in *Aspergillus* section *Fumigati*. Front Microbiol. 2016; 6: Art. 1485. https://doi.org/10.3389/fmicb.2015.01485 PMID: 26779142

2. Barrs VR, Beatty JA, Dhand NK, Talbot JJ, Bell E, Abraham LA, et al. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. Vet J. 2014; 201(2): 215–222. https://doi.org/10.1016/ j.tvjl.2014.02.020 PMID: 24685469

3. Vinh DC, Shea YR, Sugui JA, Parrilla-Castellar ER, Freeman AF, Campbell JW, et al. Invasive aspergillosis due to *Neosartorya udagawae*. Clin Infect Dis. 2009; 49(1): 102–111. https://doi.org/10.1086/ 599345 PMID: 19489714

4. Barrs VR, van Doorn TM, Houbraken J, Kidd SE, Martin P, Pinheiro MD, et al. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. PLoS One. 2013; 8(6): e64871. https://doi.org/10.1371/journal.pone.0064871 PMID: 23798996

5. Alvarez-Pérez S, Mellado E, Serrano D, Blanco JL, Garcia ME, Kwon M, et al. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. J Med Microbiol. 2014; 63(Pt 4): 617–619. https://doi.org/10.1099/jmm.0.068502-0 PMID: 24469682

6. Peláez T, Alvarez-Pérez S, Mellado E, Serrano D, Valerio M, Blanco JL, et al. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. J Med Microbiol. 2013; 62(Pt 3): 474–478. https://doi.org/10.1099/jmm.0.044867-0 PMID: 23161769

7. Coelho D, Silva S, Vale-Silva L, Gomes H, Pinto E, Sarmento A, et al. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. Med Mycol. 2011; 49(7): 755–759. https://doi.org/10.3109/13693786.2011.556672 PMID: 21299372

8. Sugui JA, Peterson SW, Figat A, Hansen B, Samson RA, Mellado E, et al. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. J Clin Microbiol. 2014; 52(10): 3707–3721. https://doi.org/10.1128/JCM.01704-14 PMID: 25100816

9. Barrs VR, Halliday C, Martin P, Wilson B, Krockenberger M, Gunew M, et al. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. Vet J. 2012; 191(1): 58–64. https://doi.org/10.1016/j.tvjl.2011.02.009 PMID: 21388842

10. Barrs VR, T JJ. Feline aspergillosis. Vet Clin North Am Small Anim Pract. 2014; 44(1): 51–73. https:// doi.org/10.1016/j.cvsm.2013.08.001 PMID: 24268333

11. Whitney BL, Broussard J, Stefanacci JD. Four cats with fungal rhinitis. J Feline Med Surg. 2005; 7(1): 53–58. https://doi.org/10.1016/j.jfms.2004.02.004 PMID: 15686974

12. Furrow E, Groman RP. Intranasal infusion of clotrimazole for the treatment of nasal aspergillosis in two cats. J Am Vet Med Assoc. 2009; 235(10): 1188–1193. https://doi.org/10.2460/javma.235.10.1188 PMID: 19912040

13. Kano R, Takahashi T, Hayakawa T, Yamaya Y, Hasegawa A, Kamata H. The first case of feline sinonasal aspergillosis due to *Aspergillus fischeri* in Japan. J Vet Med Sci. 2015; 77(9): 1183–1185. https://doi. org/10.1292/jvms.14-0454 PMID: 25947561

14. Nováková A, Hubka V, Dudová Z, Matsuzawa T, Kubátová A, Yaguchi T, et al. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. Fungal Diversity. 2014; 64(1): 253–274.

15. Gonçalves VN, Cantrell CL, Wedge DE, Ferreira MC, Soares MA, Jacob MR, et al. Fungi associated with rocks of the Atacama Desert: taxonomy, distribution, diversity, ecology and bioprospection for bioactive compounds. Environ Microbiol. 2016; 18(1): 232–245. https://doi.org/10.1111/1462-2920.13005 PMID: 26235221

16. McLennan EI, Ducker SC, Thrower LB. New soil fungi from Australian heathland: *Aspergillus, Penicillium, Spegazzinia*. Aust J Bot. 1954; 2: 355–364.

17. Kidd SE, Ling LM, Meyer W, Orla Morrissey C, Chen SC, Slavin MA. Molecular epidemiology of invasive aspergillosis: lessons learned from an outbreak investigation in an Australian hematology unit. Infect Control Hosp Epidemiol. 2009; 30(12): 1223–1226. https://doi.org/10.1086/648452 PMID: 19848605

18. Houbraken J, Samson RA. Standardization of methods for detecting heat-resistant fungi. Adv Exp Med Biol. 2006; 571: 107–111. https://doi.org/10.1007/0-387-28391-9_5 PMID: 16408595

19. Samson RA, Houbraken J, Thrane U, Frisvad JC. Food and Indoor fungi. Utrecht: Centraalbureau voor Schimmelcultures; 2010.

20. White TJ, Bruns T, Lee SJ T W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A. G DH, Sninsky J. J. & White T. J., editor. PCR Protocols A Guide to Methods and Applications. New York: Academic Press; 1990. pp. 315–322.

21. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol. 1995; 61(4): 1323–1330. PMID: 7747954

22. National Centre for Biotechnology Information. Basic Local Alignment Search Tool (BLAST): Standard Nucleotide BLAST 2016 [cited 2016 15 July]. http://blast.ncbi.nlm.nih.gov/Blast.cgi.

23. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013; 30: 2725–2729. https://doi.org/10.1093/molbev/mst197 PMID: 24132122

24. Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia. 2005; 97(6): 1316–1329. PMID: 16722222

25. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics Applications Note. 2006; 22(21): 2688–2690.

26. Ronquist F, Huelsenbecj J, Teslenko M. MrBayes version 3.2.1 http://mrbayes.sourceforge.net/2011 [cited 2017 January].

27. Frisvad JC, Samson RA. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate *Penicillia* and their mycotoxins. Stud Mycol 2004; 49: 1–173.

28. Hocking AD, Pitt JI. Fungi and Food Spoilage. Third ed. New York: Springer Science + Business Media LLC; 2009.

29. Smedsgaard J. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A. 1997; 760(2): 264–270. PMID: 9062989

30. Nielsen ML, Nielsen JB, Rank C, Klejnstrup ML, Holm DK, Brogaard KH, et al. A genome-wide polyketide synthase deletion library uncovers novel genetic links to polyketides and meroterpenoids in *Aspergillus nidulans*. FEMS Microbiol Lett. 2011; 321(2):157–66. https://doi.org/10.1111/j.1574-6968.2011. 02327.x PMID: 21658102

31. Talbot JJ, Kidd SE, Martin P, Beatty JA, Barrs VR. Azole resistance in canine and feline isolates of *Aspergillus fumigatus*. Comp Immunol Microbiol Infect Dis. 2015; 42: 37–41. https://doi.org/10.1016/j. cimid.2015.08.002 PMID: 26387063

32. O'Gorman CM, Fuller HT, Dyer PS. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. Nature. 2009; 457: 471–475. https://doi.org/10.1038/nature07528 PMID: 19043401

33. Matsuzawa T, Takaki GMC, Yaguchi T, Okada K, Abliz P, Gonoi T, et al. *Aspergillus arcoverdensis*, a new species of *Aspergillus* section *Fumigati* isolated from caatinga soil in State of Pernambuco, Brazil. Mycoscience. 2015; 56(2): 123–131.

34. Alastruey-Izquierdo A, Mellado E, Peláez T, Pemán J, Zapico S, Alvarez M, et al. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). Antimicrob Agents Chemother. 2013; 57(7): 3380–3387. https://doi.org/10.1128/AAC.00383-13 PMID: 23669377

35. Balajee SA, Kano R, Baddley JW, Moser SA, Marr KA, Alexander BD, et al. Molecular identification of *Aspergillus* species collected for the transplant-associated infection surveillance network. J Clin Microbiol. 2009; 47(10): 3138–3141. https://doi.org/10.1128/JCM.01070-09 PMID: 19675215

36. Negri CE, Gonc¸alves SS, Xafranski H, Bergamasco MD, Aquino VR, Castro PT, et al. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. J Clin Microbiol. 2014; 52(10): 3633–3640. https://doi.org/10.1128/JCM.01582-14 PMID: 25078909

37. Sabino R, Veríssimo C, Parada H, Brandão J, Viegas C, Carolino E, et al. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. Med Mycol. 2014; 52(5):519–529. https://doi.org/10.1093/mmy/myu006 PMID: 24847040

38. Escribano P, Peláez T, Muñoz P, Bouza E, Guinea J. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? Antimicrob Agents Chemother. 2013; 57(6):2815–2820. https://doi.org/10.1128/ AAC.02487-12 PMID: 23629706

39. Whitney JL, Krockenberger MB, Day MJ, Beatty JA, Dhand NK, Barrs VR. Immunohistochemical Analysis of Leucocyte Subsets in the Sinonasal Mucosa of Cats with Upper Respiratory Tract Aspergillosis. J Comp Pathol. 2016; 155(2–3): 130–140. https://doi.org/10.1016/j.jcpa.2016.08.001 PMID: 27576043

40. Sylvain ZA, Wall DH. Linking soil biodiversity and vegetation: implications for a changing planet. Am J Bot. 2011; 98(3): 517–527. https://doi.org/10.3732/ajb.1000305 PMID: 21613143

41. Greene RSB, Chartres CJ, Hodgkinson KH. The effect of fire on the soil in a degraded semi-arid woodland. I. Cryptogram cover and physical and micromorphological properties. Aust J Soil Res. 1990; 28: 755–777.

42. Eldridge DJ, Greene RSB. Microbiotic soil crusts: a review of their roles in soil and ecological processes in the rangelands of Australia. Aust J Soil Res. 1994; 32: 389–415.

43. Ni M, Feretzaki M, Sun S, Wang X, Heitman J. Sex in Fungi. Annu Rev Genet. 2011; 45: 405–430. https://doi.org/10.1146/annurev-genet-110410-132536 PMID: 21942368

44. Samson RA, Hong S, Peterson SW, Frisvad JC, Varga J. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. Stud Mycol. 2007; 59: 147–203. https://doi.org/10.3114/sim. 2007.59.14 PMID: 18490953

45. Panda A, Ghosh AK, Mirdha BR, Xess I, Paul S, Samantaray JC, et al. MALDI-TOF mass spectrometry for rapid identification of clinical fungal isolates based on ribosomal protein biomarkers. J Microbiol Methods. 2015; 109: 93–105. https://doi.org/10.1016/j.mimet.2014.12.014 PMID: 25541362

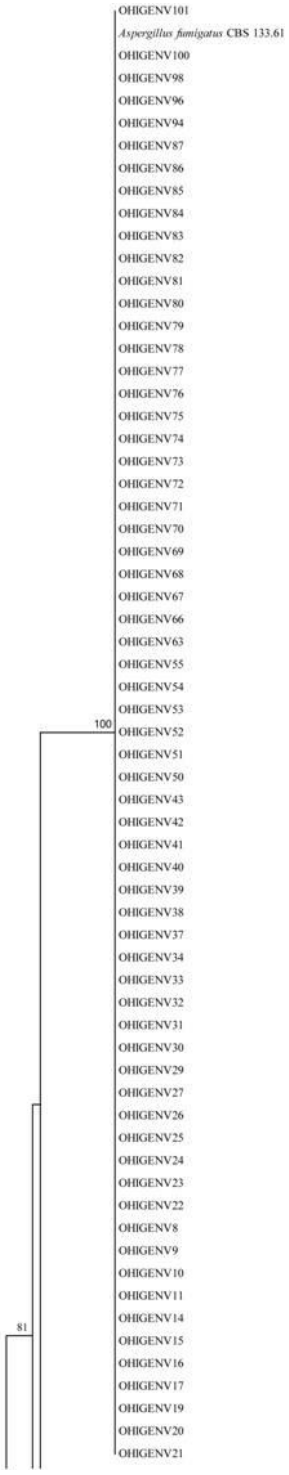
46. Hong SB, Shin HD, Hong J, Frisvad JC, Nielsen PV, Varga J, et al. New taxa of *Neosartorya* and *Aspergillus* in *Aspergillus* section *Fumigati*. Antonie Van Leeuwenhoek. 2008; 93(1–2): 87–98. https://doi.org/10.1007/s10482-007-9183-1 PMID: 17610141

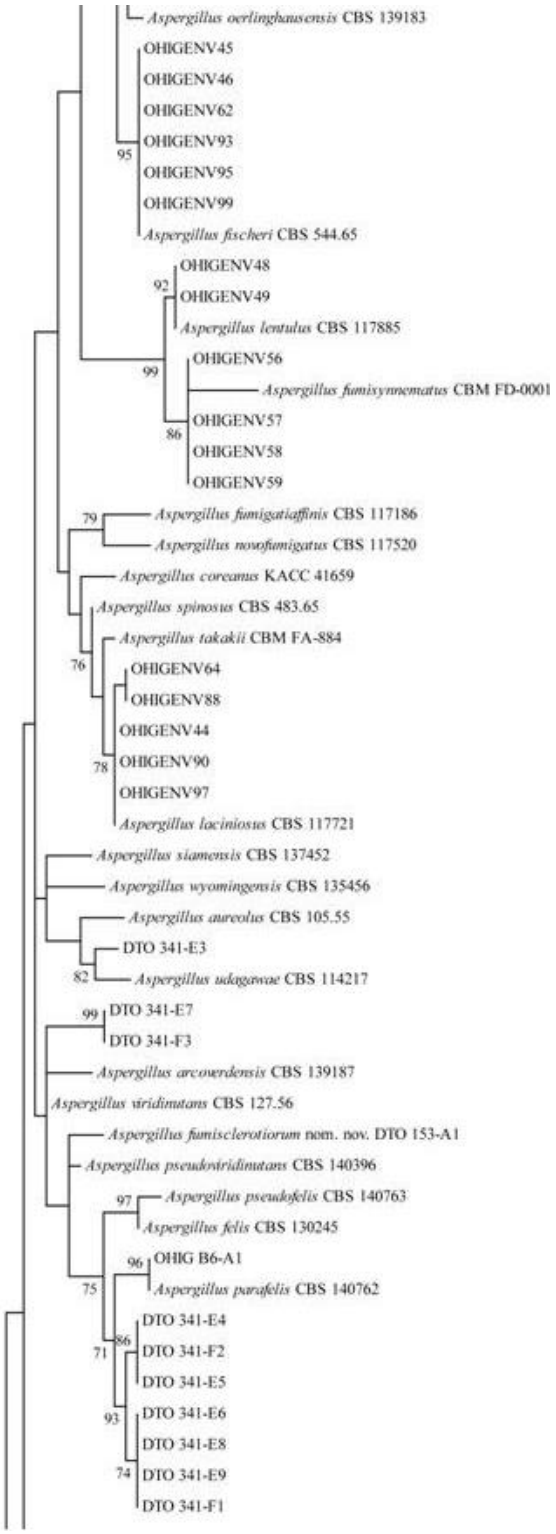
47. Gomes NM, Bessa LJ, Buttachon S, Costa PM, Buaruang J, Dethoup T, et al. Antibacterial and antibiofilm activities of tryptoquivalines and meroditerpenes isolated from the marine-derived fungi *Neosartorya paulistensis, N. laciniosa, N. tsunodae,* and the soil fungi *N. fischeri* and *N. siamensis*. Mar Drugs. 2014; 12(2): 822–839. https://doi.org/10.3390/md12020822 PMID: 24477284

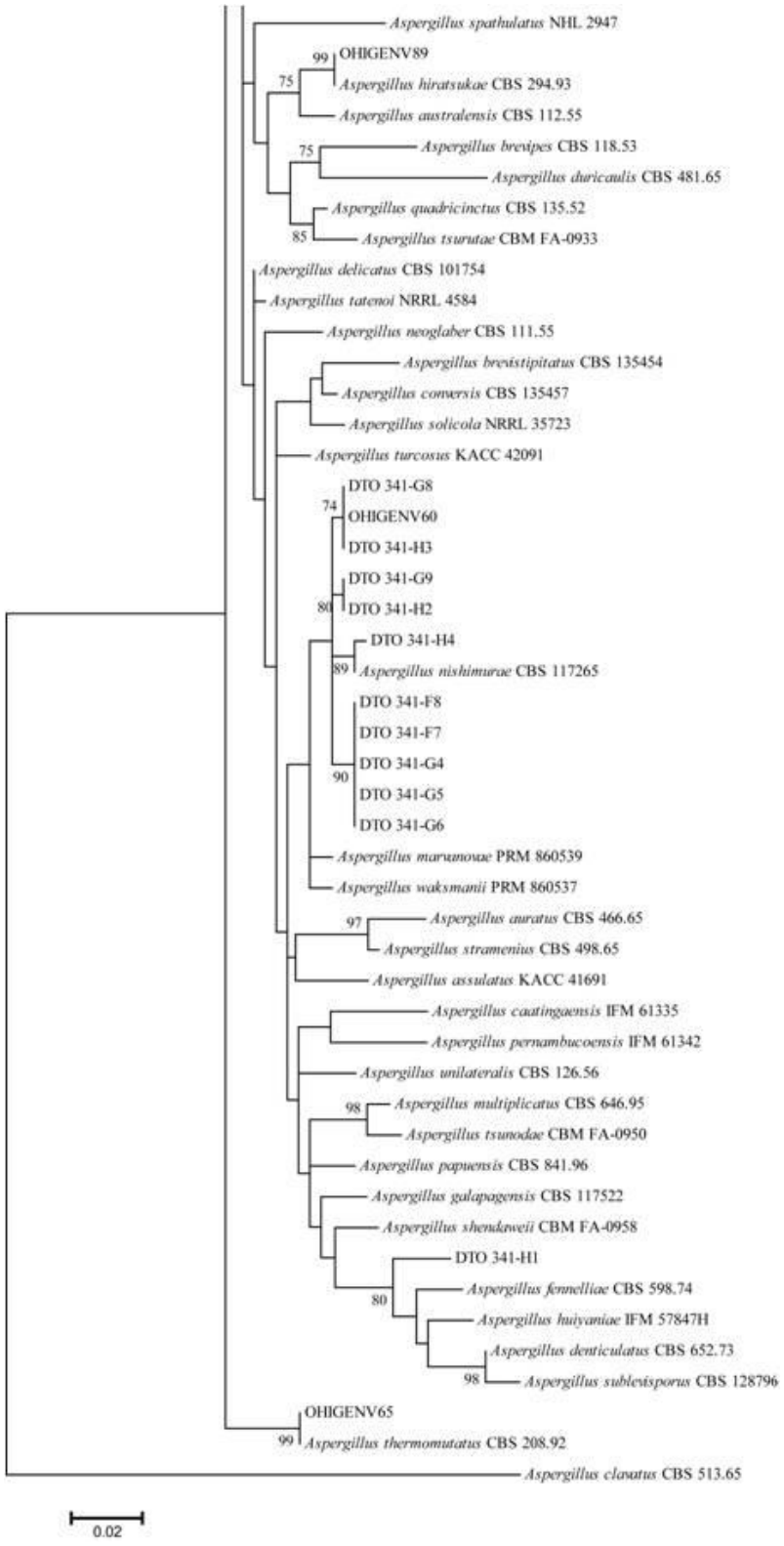
48. Pildain MB, Frisvad JC, Vaamonde G, Cabral D, Varga J, Samson RA. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. Int J Syst Evol Microbiol. 2008; 58(Pt 3): 725–735. https://doi.org/10.1099/ijs.0.65123-0 PMID: 18319485

Publication supplementary materials

S1 Figure. Phylogenetic trees of 104 environmental isolates and type strains for *A. section Fumigati* based on *BenA* sequencing (phylogeny model Kimura 2, gamma distribution, 1000 bootstrap replicates).




Aspergillus oerlinghausensis CBS 139183
OHIGENV45
OHIGENV46
OHIGENV62
OHIGENV93
OHIGENV95
OHIGENV99
Aspergillus fischeri CBS 544.65
OHIGENV48
OHIGENV49
Aspergillus lentulus CBS 117885
OHIGENV56
Aspergillus fumisynnematus CBM FD-0001
OHIGENV57
OHIGENV58
OHIGENV59
Aspergillus fumigatiaffinis CBS 117186
Aspergillus novofumigatus CBS 117520
Aspergillus coreanus KACC 41659
Aspergillus spinosus CBS 483.65
Aspergillus takakii CBM FA-884
OHIGENV64
OHIGENV88
OHIGENV44
OHIGENV90
OHIGENV97
Aspergillus laciniosus CBS 117721
Aspergillus siamensis CBS 137452
Aspergillus wyomingensis CBS 135456
Aspergillus aureolus CBS 105.55
DTO 341-E3
Aspergillus udagawae CBS 114217
DTO 341-E7
DTO 341-F3
Aspergillus arcoverdensis CBS 139187
Aspergillus viridinutans CBS 127.56
Aspergillus fumisclerotiorum nom. nov. DTO 153-A1
Aspergillus pseudoviridinutans CBS 140396
Aspergillus pseudofelis CBS 140763
Aspergillus felis CBS 130245
OHIG B6-A1
Aspergillus parafelis CBS 140762
DTO 341-E4
DTO 341-F2
DTO 341-E5
DTO 341-E6
DTO 341-E8
DTO 341-E9
DTO 341-F1
95
92
99
86
79
76
78
82
99
97
96
75
86
71
93
74


Aspergillus spathulatus NHL 2947
99
OHIGENV89
75
Aspergillus hiratsukae CBS 294.93
Aspergillus australensis CBS 112.55
75
Aspergillus brevipes CBS 118.53
Aspergillus duricaulis CBS 481.65
Aspergillus quadricinctus CBS 135.52
85
Aspergillus tsurutae CBM FA-0933
Aspergillus delicatus CBS 101754
Aspergillus tatenoi NRRL 4584
Aspergillus neoglaber CBS 111.55
Aspergillus brevistipitatus CBS 135454
Aspergillus conversis CBS 135457
Aspergillus solicola NRRL 35723
Aspergillus turcosus KACC 42091
DTO 341-G8
74
OHIGENV60
DTO 341-H3
DTO 341-G9
80
DTO 341-H2
DTO 341-H4
89
Aspergillus nishimurae CBS 117265
DTO 341-F8
DTO 341-F7
DTO 341-G4
90
DTO 341-G5
DTO 341-G6
Aspergillus maruanoae PRM 860539
Aspergillus waksmanii PRM 860537
97
Aspergillus auratus CBS 466.65
Aspergillus stramenius CBS 498.65
Aspergillus assulatus KACC 41691
Aspergillus caatingaensis IFM 61335
Aspergillus pernambucoensis IFM 61342
Aspergillus unilateralis CBS 126.56
98
Aspergillus multiplicatus CBS 646.95
Aspergillus tsunodae CBM FA-0950
Aspergillus papuensis CBS 841.96
Aspergillus galapagensis CBS 117522
Aspergillus shendaweii CBM FA-0958
DTO 341-H1
Aspergillus fennelliae CBS 598.74
80
Aspergillus huiyaniae IFM 57847H
Aspergillus denticulatus CBS 652.73
98
Aspergillus subalbisporus CBS 128796
OHIGENV65
99
Aspergillus thermomutatus CBS 208.92
Aspergillus clavatus CBS 513.65
0.02

S1 Table. *Aspergillus* sect. *Fumigati* species isolated from air and diluted, heat-treated soil from eight Australian properties where cats were domiciled with confirmed feline upper respiratory tract aspergillosis due to AVSC and one National Park site. (#), number of isolates found at property.

Property 1 (Leichhardt, NSW)	**Property 2 (Lane Cove, NSW)**	**Property 3 (Anna Bay, NSW)**	**Property 4 (Russel Lea, NSW)**	**Property 5 (Amaroo, ACT)**	**Property 6 (Miranda, NSW)**	**Property 7 (Lara, VIC)**	**Property 8 (Kealba, VIC)**	**Reserve site (Frankston, VIC)**
A. fumigatus (5) *A. fischeri* (5) *A. laciniosus* (2)	*A. fumigatus* (4) *A. lentulus* (1)	*A. fumigatus* (1) *A. fumisynnematus* (4) *A. fennelliae* (1)	*A. fumigatus* (3) *A. fischeri* (1) *A. lentulus* (1) *A. laciniosus* (1) *A. marvanovae* (3) *A. nishimurae* (6)	*A. laciniosus* (2) *A. hiratsukae* (1)	*A. fumigatus* (6)	*A. fumigatus* (5)	*A. thermomutatus* (1) *A. fumigatus* (27) *A. marvanovae* (2)	*A. fumigatus* (13) *A. felis*-clade (7) *A. frankstonensis* (2)

S2 Table. AVSC species isolates that were paired with *A. frankstonensis* sp. nov. DTO 341-F3 (MAT1-2).

Mating type	**Species**	**ID number**
MAT1-1	*A. felis*-clade	DTO 341-F1
MAT1-1	*A. felis*-clade	DTO 341-E6
MAT1-1	*A. felis*-clade	DTO 341-E4
MAT1-1	*A. felis*-clade	DTO 341-E8
MAT1-1	*A. felis*-clade	DTO 341-E9
MAT1-1	*A. felis*	DTO 131-E3
MAT1-1	*A. felis*	DTO 131-E5
MAT1-1	*A. viridinutans*	IFM 47045
MAT1-1	*A. udagawae*	IFM 46972
MAT1-1	*A. arcoverdensis*	IFM 61334
MAT1-1	*A. felis*-clade	NRRL 62901

3.4 Conclusions

The publication in this chapter demonstrates the variety of *Aspergillus* species pathogens isolated from Australian soil and air from areas where humans and animals cohabitate. I showed a high incidence of pathogenic species other than *A. fumigatus* in samples from domestic environments, including isolates known to cause feline aspergillosis.

Invasive fungal rhinosinusitis is a chronic disease in cats (Barrs et al., 2012). A limitation to this type of research is that most cats have advanced disease at diagnosis and many were deceased at the time of environmental sampling, so the source of infection may have been long since removed from their environment. This makes identification of control measures for disease prevention difficult to determine. Since *Aspergillus* conidia are dispersed widely through air, prevention measures may be difficult. Suggesting pre-disposed cat breeds be indoor only would be unlikely to prevent disease as isolates of *A. felis* have been sampled from indoor environments (Barrs et al., 2013). Clearly *A. felis* resides in soil and is dispersed through the air (Nováková et al., 2014, Barrs et al., 2013). It is likely that one way cats are exposed to this fungus is when they interact with soils, such as scratching and digging associated with toileting behaviours. It is sensible to suggest that when removing soil and laying down new soil, mulches or composts, that feline access to these areas be restricted, especially in breeds that are predisposed to fungal infection, to avoid increased environmental exposure to fungal spores.

This publication demonstrated the AVSC members *A. felis, A. parafelis* and *A. pseudofelis* are all closely related and likely the same species, and it was suggested the name *A. felis*-clade be used when referring to these species until further taxonomic analysis resolves this. Preliminary published results by another research group also support these findings, and additionally this work has identified another likely new species within the complex, previously identified as *A. aureolus* species and temporarily called *A. acrensis* (Lyskova et al., 2018). Further taxonomic analysis being undertaken with Vit Hubka, a mycology taxonomist and PhD candidate in the Department of Botany at Charles University, Prague, includes the isolates from this chapter.

Interestingly, we isolated *A. felis*-clade species from woodland soil from a nature reserve in Frankston where the founding species of the *Aspergillus viridinutans* species complex, *A. viridinutans* sensu stricto, was isolated in the 1950s (McLennan et al., 1954). We also found a new *A. viridinutans* species complex member, *A. frankstonensis* in the same location, and this species is most closely related to *A. viridinutans*. It appears that the environment at Frankston has offered a good ecological niche for members of the
A. viridinutans species complex for a considerable time. Another study found *A. felis* associated with rocks in the Atacama Desert in Chile, offering a contrasting ecology to Frankston (Gonçalves et al., 2016). *A. viridinutans* species complex members thrive best at temperatures between 37- 45 ° Celsius on Malt Extract Agar (McLennan et al., 1954, Fennell and Raper, 1955, Horie et al., 1995, Samson et al., 2007, Barrs et al., 2013, Eamvijarn et al., 2013, Howard SJ, 2014, Samson et al., 2014, Nováková et al., 2014, Matsuzawa et al., 2015) but their ascospore form can withstand more extreme temperatures (Barrs et al., 2013, Nováková et al., 2014, Sugui et al., 2014). Their special requirements for growth in the laboratory may support their predilection for a particular ecological niche.

This publication isolated many environmental *A. fumigatus* isolates. Considering the growing global prevalence of ARAf, I was interested in further examining the antifungal susceptibility of these environmental isolates, and this is the subject of Chapter 6. I was also interested in further investigating the antifungal susceptibilities and *cyp51A* sequences of environmental versus clinical AVSC isolates to determine if higher MICs for azoles, generally observed amongst members of this species complex, could be attributed to mutations in this gene, commonly observed amongst azole-resistant *A. fumigatus*, and this is further explored in a subsequent chapter (Chapter 7).

3.5 References

BARRS, V. R., HALLIDAY, C., MARTIN, P., WILSON, B., KROCKENBERGER, M., GUNEW, M., BENNETT, S., KOEHLMEYER, E., THOMPSON, A., FLIEGNER, R., HOCKING, A., SLEIMAN, S., O'BRIEN, C. & BEATTY, J. A. 2012. Sinonasal and sino-

orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *The Veterinary Journal,* 191**,** 58-64.

BARRS, V. R., VAN DOORN, T. M., HOUBRAKEN, J., KIDD, S. E., MARTIN, P., PINHEIRO, M. D., RICHARDSON, M., VARGA, J. & SAMSON, R. A. 2013. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One,* 8**,** e64871.

EAMVIJARN, A., MANOCH, L., CHAMSWARNG, C., PIASAI, O., VISARATHANONTH, N., LUANGSA-ARD, J. J. & KIJJOA, A. 2013. *Aspergillus siamensis* sp. nov. from soil in Thailand. *Mycoscience,* 54**,** 401-405.

FENNELL, D. I. & RAPER, K. B. 1955. New species and varieties of *Aspergillus*. *Mycologia,* 47**,** 71-75.

GLASS, N. L. & DONALDSON, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology,* 61**,** 1323-1330.

GONÇALVES, V. N., CANTRELL, C. L., WEDGE, D. E., FERREIRA, M. C., SOARES, M. A., JACOB, M. R., OLIVEIRA, F. S., GALANTE, D., RODRIGUES, F., ALVES, T. M., ZANI, C. L., JUNIOR, P. A., MURTA, S., ROMANHA, A. J., BARBOSA, E. C., KROON, E. G., OLIVEIRA, J. G., GOMEZ-SILVA, B., GALETOVIC, A., ROSA, C. A. & ROSA, L. H. 2016. Fungi associated with rocks of the Atacama Desert: taxonomy, distribution, diversity, ecology and bioprospection for bioactive compounds. *Environmental Microbiology,* 18**,** 232-245.

HONG, S. B., GO, S. J., SHIN, H. D., FRISVAD, J. C. & SAMSON, R. A. 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia,* 97**,** 1316-1329.

HORIE, Y., MIYAJI, M., NISHIMURA, K., FRANCO, M. F. & COELHO, K. L. R. 1995. New and interesting species of *Neosartorya* from Brazilian soil. *Mycoscience,* 36**,** 199-204.

HOUBRAKEN, J. & SAMSON, R. A. 2006. Standardization of methods for detecting heat-resistant fungi. *Advances in Experimental Medicine and Biology,* 571.

HOWARD SJ 2014. Multi-resistant aspergillosis due to cryptic species. *Mycopathologia,* 178**,** 435–439.

KIDD, S. E., LING, L. M., MEYER, W., ORLA MORRISSEY, C., CHEN, S. C. & SLAVIN, M. A. 2009. Molecular epidemiology of invasive aspergillosis: lessons learned from an outbreak investigation in an Australian hematology unit. *Infect Control Hosp Epidemiol,* 30**,** 1223-6.

LYSKOVA, P., HUBKA, V., SVOBODOVA, L., BARRS, V., DHAND, N. K., YAGUCHI, T., MATSUZAWA, T., HORIE, Y., KOLARIK, M., DOBIAS, R. & HAMAL, P. 2018. Antifungal susceptibility of the *Aspergillus viridinutans* complex: comparison of two *in vitro* methods. *Antimicrobial Agents and Chemotherapy,* 62.

MATSUZAWA, T., TAKAKI, G. M. C., YAGUCHI, T., OKADA, K., ABLIZ, P., GONOI, T. & HORIE, Y. 2015. *Aspergillus arcoverdensis*, a new species of *Aspergillus* section *Fumigati* isolated from caatinga soil in State of Pernambuco, Brazil. *Mycoscience,* 56**,** 123-131.

MCLENNAN, E. I., DUCKER, S. C. & THROWER, L. B. 1954. New soil fungi from Australian heathland: *Aspergillus, Penicillium, Spegazzinia*. *Australian Journal of Botany,* 2**,** 355-364.

NIELSEN, M. L., NIELSEN, J. B., RANK, C., KLEJNSTRUP, M. L., HOLM, D. K., BROGAARD, K. H., HANSEN, B. G., FRISVAD, J. C., LARSEN, T. O. & MORTENSEN, U. H. 2011. A genome-wide polyketide synthase deletion library uncovers novel genetic links to polyketides and meroterpenoids in *Aspergillus nidulans*. *FEMS Microbiology Letters,* 321**,** 157-166.

NOVÁKOVÁ, A., HUBKA, V., DUDOVÁ, Z., MATSUZAWA, T., KUBÁTOVÁ, A., YAGUCHI, T. & KOLAŘÍK, M. 2014. New species in *Aspergillus* section *Fumigati* from

reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. *Fungal Diversity.,* 64**,** 253-274.

RONQUIST, F., HUELSENBECJ, J. & TESLENKO, M. 2011. *MrBayes version 3.2.1* [Online]. http://mrbayes.sourceforge.net/. [Accessed January 2017].

SAMSON, R. A., HONG, S., PETERSON, S. W., FRISVAD, J. C. & VARGA, J. 2007. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Studies in Mycology,* 59**,** 147-203.

SAMSON, R. A., HOUBRAKEN, J., THRANE, U. & FRISVAD, J. C. 2010. *Food and Indoor fungi,* Utrecht, Centraalbureau voor Schimmelcultures.

SAMSON, R. A., VISAGIE, C. M., HOUBRAKEN, J., HONG, S. B., HUBKA, V., KLAASSEN, C. H., PERRONE, G., SEIFERT, K. A., SUSCA, A., TANNEY, J. B., VARGA, J., KOCSUBÉ, S., SZIGETI, G., YAGUCHI, T. & FRISVAD, J. C. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Studies in Mycology,* 78**,** 141-173.

SMEDSGAARD, J. 1997. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *Journal of Chromatography. A,* 760**,** 264-270.

STAMATAKIS, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics Applications Note,* 22**,** 2688-2690.

SUGUI, J. A., PETERSON, S. W., FIGAT, A., HANSEN, B., SAMSON, R. A., MELLADO, E., CUENCA-ESTRELLA, M. & KWON-CHUNG, K. J. 2014. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J Clin Microbiol,* 52**,** 3707-3721.

WHITE, T. J., BRUNS, T. & LEE, S. J. W. T. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* M. A. INNIS, D. H. G., J. J. SNINSKY & T. J. WHITE, (ed.) *PCR Protocols. A Guide to Methods and Applications.* New York: Academic Press.

Chapter 4. Agents of avian aspergillosis amongst captive birds in Australia

4.1 Introduction

Birds in captive settings are particularly susceptible to developing aspergillosis. Having performed an Australian environmental investigation for *Aspergillus felis*-clade species, I wanted to next investigate isolates of *Aspergillus* species sampled from birds in Australia, to determine if, in addition to domestic small animals (cat and dogs), these or other cryptic *Aspergillus* species were causing disease in birds.

4.2 Premise

The publication in this chapter includes a prospective investigation of the species causing aspergillosis in captive birds in Australia. This research was undertaken to determine if cryptic and/or common *Aspergillus* species were causing avian disease, as captive birds are highly susceptible to aspergillosis, commonly attributed to high environmental fungal burden and host stress/ immunosuppression. If birds were being infected with cryptic species, then it was highly likely these fungi would be readily isolated from their environment, possibly shedding further light on disease pathogenesis.

Global surveillance studies using multi-locus identification of avian *Aspergillus* isolates were lacking in the literature, with existing molecular studies from Europe, the USA, South America, China and Australia only performing genotyping *A. fumigatus* sensu stricto using specific microsatellite markers, or one gene (Katz et al., 1996, Lair-Fulleringer et al., 2003, Beernaert et al., 2009, Thierry et al., 2010, Lança et al., 2010, Olias et al., 2011, Van Waeyenberghe et al., 2011, Burco et al., 2012, Wang et al., 2014, Li et al., 2015, de Oca et al., 2017).

The publication in this chapter demonstrates the fungal species causing disease in captive birds in Australia are similar to those of humans and dogs where infection due to *A. fumigatus* is most common (Balajee et al., 2009, Alastruey-Izquierdo et al., 2013, Negri et al., 2014, Sabino et al., 2014, Talbot et al., 2014), contrasting with Australian cats with sino-orbital aspergillosis,

where cryptic *Aspergillus* species are commonly the cause of infection (Kano et al., 2008, Barrs et al., 2012, Barrs et al., 2013, Kano et al., 2013, Barrs et al., 2014, Barrs and Talbot, 2014).

To perform the study, isolates were collected and processed over a three year period (2014 to 2017). Included fungal cultures had been identified phenotypically as *Aspergillus* species by veterinary microbiologists prior to submission to the study. Molecular identification was performed for each sample, as is recommended for identification of clinical isolates to genus and species level (Balajee et al., 2007), including amplification of the ITS region (White et al., 1990) and partial beta-tubulin gene (Glass and Donaldson, 1995). All sequences were deposited in GenBank (National Center for Biotechnology Information, 2016). Where available, clinical history and post mortem and histopathology reports were forwarded to me for analysis of infection type. Selected isolates were deposited into the Mycobank private collection of Prof. Vanessa Barrs at the University of Sydney, with some studied further in Chapter 6.

4.3 Publication

The following publication has been re-formatted here to suit the style of this thesis. The publication's in-text references remain in the style required by the journal (superscript number form) and the publication associated tables, figures, references and supplementary data follow the main text. This research is presented in its original publication form in **Appendix 3**. A list of all other references referred to in this chapter outside of section **4.3 Publication** can be found at the chapter's end (see **4.5 References**).

Talbot JJ, Thompson P, Vogelnest L, Barrs VR. Identification of pathogenic *Aspergillus* isolates from captive birds in Australia. *Medical Mycology.* 2017; myx137, https://doi.org/10.1093/mmy/myx137.

Abstract

Aspergillosis is a major cause of severe respiratory disease in birds. The prevalence of cryptic section *Fumigati* and other non–*Aspergillus fumigatus* species as causative agents is unknown. Species identity was determined in 30 isolates from affected birds from zoos, pet birds and poultry by PCR of the ITS1-5.8S-ITS2 and partial β-tubulin genes. The most prevalent isolate

was *A. fumigatus* sens. str. in 87% (26) cases. Other *Aspergillus* species were identified in 13% (4) cases, including *A. restrictus* (1), *A. flavus* sens. str. (2), and *A. nidulans*-clade (1). This is the first report of *A. restrictus* causing avian disease.

Introduction

Avian aspergillosis, first reported in the 1800s,[1] is a significant cause of mortality among captive birds worldwide. It most commonly causes disease within the respiratory tract, and can progress to disseminated invasive aspergillosis. Two forms of lesions have been described; a granulomatous deep nodular form affecting non-aerated parenchyma, and a pyogranulomatous nonencapsulated superficial diffuse form involving lungs and serosae.[2]

Captive birds are highly susceptible to aspergillosis due to environmental factors (increased burden of fungal spores), host immune factors (immunosuppression from disease, stress, or therapeutics), and unique respiratory anatomy.[1,2] Anatomical differences to mammals that enable unique airflow in birds include nonexpansile and unlobed lungs with no pleural cavity, looped parabronchi with bi-directional air flow capillaries instead of terminal ending alveoli, and cranial and caudal air sacs that extend the bronchial system beyond the lung to pneumatize bones and enable fresh airflow during inspiration and expiration.[3] Infection occurs when innate defenses cannot eliminate spores in parabronchial air capillaries, resulting in fungal plaques that can penetrate surrounding tissues.[2] Despite a range of antifungal therapeutic treatment options, infections are often fatal.[2]

The clinical importance of fungal molecular identification techniques has been demonstrated for human and nonavian animal species with aspergillosis, with cryptic section *Fumigati* species increasingly identified, prompting epidemiological studies.[4–9] We previously identified isolates causing respiratory aspergillosis in cats and dogs and found that disease in dogs, like humans is predominantly caused by *A. fumigatus* sens. str.,[8] while in cats, infections with cryptic species in section *Fumigati* including *A. felis, A. udagawae,* and *A. thermomutatus* are most prevalent.[9] We performed environmental sampling for *Aspergillus* section *Fumigati* in homes of cats previously diagnosed with aspergillosis and in a recreational nature reserve and found that cryptic species reported to cause disease in humans or animals were common, comprising 25% of isolates overall.[10]

Aspergillus fumigatus sens. str. has been most commonly associated with avian infections based on phenotypic identification techniques, with *A. flavus, A. niger, A. glaucus,* and *A. nidulans* also reported.[2] Thus, cryptic species may have been overlooked as a cause of avian aspergillosis.

This study aimed to definitively identify fungal pathogens causing avian aspergillosis and to determine the prevalence of cryptic *A.* section *Fumigati* species. A secondary aim was to determine the form of aspergillosis present.

Methods

Australian avian veterinarians were contacted to recruit isolates from clinical cases of avian aspergillosis between 2013 and 2017. *Aspergillus i*solates cultured from lesions in captive birds consistent with aspergillosis, including fungal plaques or granulomas observed within the respiratory tract or other parenchyma during diagnostic investigation or at post mortem were included for study. Morphological identification of *Aspergillus* species was performed by microbiologists, based on consistent macroscopic and microscopic features including identification of smooth or rough walled conidia stemming from radiate or columnar conidial heads with globose or subclavate vesicles, stipe and a foot cell.[11] Thirty isolates were received from Australian native and exotic avian species (Table 1), including 26 from zoos (New South Wales [*n* = 23, Taronga Conservation Society approved opportunistic sampling request R14B178], South Australia [*n* = 3]), one from private practice (Queensland) and three from commercial poultry producers (NSW). Histopathology post-mortem reports were available for review in 20 zoological cases.

All isolates were subcultured onto malt extract agar and grown in the dark at 25°C for up to 7 days until sporulation occurred. DNA was extracted using the MoBio DNA isolation kit (QIAGEN MO BIO, Carlsbad, USA). Conventional polymerase chain reaction (PCR) was performed targeting the rDNA gene cluster, including the ITS1, 5.8S gene, and ITS2 regions using primers ITS1 and ITS4.[12] Amplification of the partial β-tubulin (BenA) gene was performed using primers bt2a and bt2b.[13] PCR product was sent to an external laboratory for purification and Sanger sequencing (Macrogen, Seoul, South Korea). Generated sequences were edited using BioEdit version 7.2.5.[14] Species identification was determined using NCBI-BLAST tool and sequence homology of ≥99% (ITS) and ≥99.5% (BenA) with type strains

sequences.[15] If BenA homology was <99.5%, a separate alignment and phylogenetic analysis was performed in Mega 6.06[16] with sequences of all section type-strains to confirm the closest type-strain match using the maximum likelihood method based on the Tamura-Nei model with gamma distribution and 1000 bootstrap replicates. Sequences were deposited in GenBank (MF540250-MF540309).

Results

Twenty-six of 30 (87%) isolates were identified as *A. fumigatus* sens. str., and the remaining four (13%) were other *Aspergillus* species, including *A. restrictus* (*n* = 1), *A. flavus* sens. str. (*n* = 2), and *A. nidulans*-clade (*n* = 1) (Table 1). The latter isolate had 99.1% BenA homology with *A. nidulans* sens. str. Phylogenetic analysis with section *Nidulantes* type-strains placed this isolate in the *A. nidulans*-clade, where it was most closely related to *A. pachychristatus* and *A. latilabiatus*.

Affected avian species were 70% native and 30% exotic to Australia; passerines were most commonly affected (30%). Five *A. fumigatus* sens. str. isolates were from IUCN listed critically endangered native species (http://www.iucnredlist.org/search) including Plains Wanderer (*Pedionomus torquatus*) (3), Regent Honeyeater (*Anthochaera Phrygia*) (1), and Swift Parrot (*Lathamus discolor*) (1).

Histopathology findings are summarized in supplementary Table 1. Seventeen of 20 cases had invasive aspergillosis, which was disseminated in 12 and focal in five (respiratory). There were three cases of noninvasive localized infection, involving conjunctiva, air sacs, and a coelomic cavity mass. All six respiratory cases (5 invasive, 1 noninvasive) involved the lower respiratory tract; localized to the air sacs (1), the lungs (4), or both air sacs and lungs (1). In four cases, a primary nonfungal disease process was identified including mycobacteriosis, egg yolk peritonitis, metastatic pancreatic adenocarcinoma and chronic liver disease.

Discussion

Aspergillus fumigatus sens. str. was the most common isolate amongst our study population (87%), similar to prevalence studies in humans, dogs, and the environment.[4–8] While other *Aspergillus* species were identified in 13% of cases, no cryptic section *Fumigati* species were

identified. This is in contrast to prevalence studies in humans (0.75–3.7% of cases)[4–6] and reports on cats, in which invasive fungal rhinosinusitis is the most common form and is caused by cryptic species in section *Fumigati*.[9] Although our sample size was small, we found a similar prevalence for *A. flavus* sens. str. (6%) and *A. nidulans*-clade (3%) as is reported in humans (*A. flavus* 8–13%; *A. nidulans* 0.5–2.5%).[4–6]

Our study is the first to our knowledge to report aspergillosis in birds due to *A. restrictus.* This case was in a Java Finch (*Lonchura oryzivora*) with chronic liver disease and disseminated invasive aspergillosis affecting air sacs, lung, and gizzard. *Aspergillus restrictus* has also been isolated from humans with disseminated infection following a heart valve replacement, although only phenotypic identification was performed.[17,18] Our findings support an opportunistic pathogen role for this fungal species.

This study demonstrates the value of molecular characterization of avian fungal isolates. *Aspergillus* species other than *A. fumigatus* sens. str. should also be considered as potential agents of this disease and may have important implications for patient management as seen in humans and other animals. Different *Aspergillus* species isolated from birds have been shown to have different susceptibilities to commonly used antifungal therapies, including itraconazole and amphotericin B.[19] This information could prove invaluable in management of a disease that is already challenged by lack of knowledge in avian pharmacokinetics and late-stage clinical presentation.[2]

We investigated avian aspergillosis amongst captive birds including several critically endangered native Australian species. The pathogenesis of avian disease has been associated with an increased environmental load of filamentous fungi in captive/rehabilitation settings (5.7% of fungal isolates grown) compared to natural avian habitats (0.03%),[20] with an *A. fumigatus* average air count nine times greater than natural habitats reported from rehabilitation centres.[21] Furthermore, genotyping of clinical and environmental isolates has demonstrated that the rehabilitation setting is a source of clinical disease in captive birds, likely due to this increased fungal burden and the presence of existing disease and/or stress.[21] This highlights the importance of environmental control strategies to reduce the risk of aspergillosis in captivity, and the importance of fungal load monitoring.

Overall, the diversity of *Aspergillus* genus species causing infection in captive Australian birds was limited. Surveillance of wild birds may also be important for both human and animal health, as wild animal populations can be sentinels for infectious disease.[22]

Acknowledgements

The authors wish to thank veterinary pathologists Dr Lydia Tong, Dr Karrie Rose, and Dr Cheryl Sangster at Taronga Conservation Society, Mosman, New South Wales for performing histopathology, and veterinary clinicians Dr David McLelland and Dr Jenny McLelland at Adelaide Zoo, Adelaide, South Australia, and Dr Adrian Gallagher, Brisbane Bird Vet, Chermside, Queensland for providing samples.

Publication Table

Table 1. Fungal molecular identification (internal transcribed spacer (ITS), partial beta-tubulin [BenA] gene), avian species and origin details for fungal isolates used in this study (*n*= 30), collected from 2013 to 2017. Molecular identification is based on homology percentage matches of ≥99% (ITS) and ≥99.5% (BenA) using NCBI-BLAST tool.

Fungal species	Genbank accession number (ITS; BenA)	Avian species (native/exotic)	Organ isolated from	Location
A. fumigatus sens.st.	MF540280; MF540250	Pacific Black Duck (n)	Lung	Zoo, NSW
	MF540281; MF540251	Domestic Turkey (e)	Ocular	Zoo, NSW
	MF540282; MF540252	Diamond Firetail Finch (n)	Lung	Zoo, NSW
	MF540303; MF540273	Red-browed finch (n)	Liver	Zoo, NSW
	MF540284; MF540254	Crimson Finch (n)	Lung	Zoo, NSW
	MF540283; MF540253	Metallic Starling (n)	Lung	Zoo, NSW
	MF540287; MF540257	Metallic Starling (n)	Liver	Zoo, NSW
	MF540294; MF540264	Metallic Starling (n)	Lung	Zoo, NSW
	MF540285; MF540255	Olive-backed Oriole (n)	Air sac	Zoo, NSW
	MF540288; MF540258	Red Jungle Fowl (e)	Lung	Zoo, NSW
	MF540296; MF540266	Red Jungle Fowl (e)	Abdominal mass	Zoo, NSW
	MF540289; MF540259	Little Penguin (n)	Lung	Zoo, NSW
	MF540292; MF540262	Domestic Chicken (e)	Lung	Poultry producer, NSW
	MF540293; MF540263	Rufous Owl (n)	Ocular	Zoo, NSW
	MF540295; MF540265	Regent Honeyeater (n)	Lung	Zoo, NSW
	MF540297; MF540267	Scaly-breasted Lorikeet (n)	Pericardium	Zoo, NSW
	MF540298; MF540268	Hardhead Duck (n)	Air sac	Zoo, NSW
	MF540299; MF540269	Blue Fronted Amazon Parrot (e)	Lung	Veterinarian, QLD
	MF540300; MF540270	Crimson Rosella (n)	Lung	Zoo, NSW
	MF540301; MF540271	Plains Wanderer (n)	Lung	Zoo, NSW
	MF540307; MF540277	Plains Wanderer (n)	Lung	Zoo, NSW
	MF540309; MF540279	Plains Wanderer (n)	Air sac	Zoo, NSW

	MF540305; MF540275	Swift Parrot (n)	Kidney	Zoo, SA
	MF540306; MF540276	Sacred Ibis (n)	Lung	Zoo, NSW
	MF540308; MF540278	Mandarin Duck (e)	Air sac	Zoo, SA
	MF540302; MF540272	Black-faced Cockatoo (n)	Air sac	Zoo, NSW
A. restrictus	MF540286; MF540256	Java Finch (e)	Lung	Zoo, NSW
A. flavus sens. str.	MF540290; MF540260	Domestic Chicken (e)	Lung	Poultry producer, NSW
	MF540291; MF540261	Domestic Chicken (e)	Lung	Poultry producer, NSW
A. nidulans-clade	MF540304; MF540274	Palm Cockatoo (n)	Tracheal mass	Zoo, SA

Abbreviations: e, exotic; n, native.

Table 2. Histopathology information for 20 cases of avian aspergillosis used in this study.

Avian species	**Sample site for culture**	**Histopathological diagnosis**	**Form of aspergillosis**	**Histopathology of fungal lesions**
Pacific Black Duck	Lung	Tuberculosis (*Mycobacterium avium*) with secondary pulmonary aspergillosis	Pulmonary aspergillomas (invasive, secondary to another disease process)	Multifocal necroinflammatory nodules with fungal hyphae and acid fast bacteria effacing lung parenchyma
Domestic Turkey	Ocular	Fungal conjunctivitis, enteric coccidiosis, metabolic bone disease	Conjunctivitis (localised, non-invasive)	Right side ocular and periocular inflammatory reaction extending from the conjunctiva with associated septate fungal hyphae in serocellular discharge
Diamond Firetail Finch	Lung	Invasive aspergillosis causing air sacculitis and coleomitis; hepatitis; enteritis due to microsporidia	Disseminated, invasive	Fibrin, inflammatory cells, fungal hyphae, necrotic debris in air sac, proventriculus and ventriculus
Metallic Starling	Lung	Invasive aspergillosis with associated pneumonia and encephalitis	Disseminated, invasive	Multifocal aspergillomas in lung and brain tissue (necrotic, inflammatory lesions with associated fungal hyphae); malacia of the brain
Crimson Finch	Lung	Invasive aspergillosis; ventricular yeasts; duodenal parasitism (cestodes)	Disseminated, invasive	Fibrin layer with fungal hyphae and inflammatory cells in lungs, kidneys, testis, ventriculus
Olive Backed Oriole	Air sac	Egg yolk peritonitis with secondary invasive aspergillosis	Disseminated, invasive, secondary to another disease process	Fibrin layer with fungal hyphae and inflammatory cells in air sacs and ventriculus

Java Finch	Lung	Chronic liver disease with secondary aspergillosis	Disseminated, invasive	Inflammatory and proliferative changes in air sacs, gizzard serosa consistent with aspergillosis
Metallic Starling	Liver	Invasive aspergillosis with secondary *Mycobacterium genevense* liver infection	Disseminated, invasive, secondary to another disease process	Multifocal necroinflammatory aspergillomas in lungs with associated fungal hyphae; fungal hyphae in liver; cerebellum focal malacia with inflammation and fungal hyphae
Jungle Fowl	Air sac	Mycotic air sacculitis	Granulomatous air sacculitis (diffuse, non-invasive)	Granulomatous inflammation with fungal hyphae
Little Penguin	Lung	Invasive aspergillosis	Disseminated, invasive	Multisystemic granulomas containing branching, septate fungal hyphae invading the parenchyma of the liver, the air sacs and peri-renal tissue
Rufous owl	Ocular orbit tissue	Intra-ocular and retrobulbar mycosis (right orbit) and severe mycotic pneumonia with secondary ovarian, splenic and renal glomeruli amyloidosis and hepatopathy	Disseminated, invasive	Lung parenchyma replaced by multiple cavitated, necroinflammatory nodules surrounded by fungal hyphae; multifocal necrotising panophthalmitis with multifocal thrombosis and intralesional fungal hyphae

Metallic Starling	Lung	Invasive aspergillosis	Pulmonary aspergillomas (diffuse, invasive)	Lung parenchyma replaced by multiple necroinflammatory nodules with central acute branching septate fungal hyphae; fungal hyphae in lumen of large blood vessels of lungs but no evidence of dissemination to other organs
Regent Honeyeater	Lung	Mycotic pneumonia with secondary hepatopathy	Pulmonary aspergillomas (diffuse, invasive)	Multifocal necroinflammatory nodules with fungal hyphae causing destruction of lung parenchyma
Jungle Fowl	Coelomic mass	Metastatic pancreatic adenocarcinoma with secondary coelomic aspergillosis	Focal, non-invasive, secondary to another disease process	Free coelomic mass with calcification, fibrin and haemorrhage
Scaly-breasted Lorikeet	Pericardium	Invasive aspergillosis	Disseminated, invasive	Pericardium and epicardium necrosis, inflammation and fibrin with fungal hyphae also invading into the superficial myocardium; lung parenchyma destruction by a focal necroinflammatory nodule with fungal hyphae present in nearby airways
Plains wanderer	Air sac	Invasive aspergillosis	Disseminated, invasive	Necroinflammatory foci with fungal hyphae invading the parenchyma of the lungs, proventriculus, spleen, liver and ovary
Crimson Rosella	Lung	Mycotic bronchopneumonia, hepatic amyloidosis, myositis	Pulmonary aspergillosis (diffuse, invasive)	Necroinflammatory foci with acute branching fungal hyphae in the parabronchi and alveoli and

				necrosis of alveolar walls
Plains Wanderer	Lung	Multicentric aspergillosis, endometriosis, arteritis, air sacculitis, polyserositis, biliary hyperplasia, hepatic necrosis, proventricular necrosis	Disseminated, invasive	Paraovarian aspergilloma with invasion of adjacent small intestine; multifocal fungal hyphae infiltration of lung parenchyma with associated tissue necrosis and inflammation
Swift Parrot	Kidney	Fungal nephritis, myocarditis, pericarditis	Aspergilloma (diffuse, invasive)	Multifocal nodular necroinflammatory lesions with associated fungal hyphae in the kidney
Mandarin Duck	Air sac	Mycotic air sacculitis	Aspergilloma (diffuse, invasive)	Severe, subacute to chronic multifocal air sacculitis with intralesional fungal hyphae; multifocal pulmonary granulomas containing acute branching septate fungal hyphae

Publication References

1. Neumann. Aspergillosis in domesticated birds. *J Comp Pathol*. 2016; **155**: 102–104.

2. Beernaert LA, Pasmans F, Van Waeyenberghe L, Haesebrouck F, Martel A. *Aspergillus* infections in birds: a review. *Avian Pathol*. 2010; **39**: 325– 331.

3. Dyce KM, Sack WO, Wensing CJG, eds. *Textbook of Veterinary Anatomy*, 3rd edn. Philadelphia: Saunders, 2002.

4. Negri CE, Gonçalves SS, Xafranski H et al. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. *J Clin Microbiol*. 2014; **52**: 3633–3640.

5. Balajee SA, Kano R, Baddley JW et al. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *J Clin Microbiol*. 2009; **47**: 3138–3141.

6. Alastruey-Izquierdo A, Mellado E, Peláez T et al. Population-based survey´ of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrob Agents Chemother*. 2013; **57**: 3380–3387.

7. Sabino R, Veríssimo C, Parada H et al. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. *Med Mycol*. 2014; **52**: 519–529.

8. Talbot JJ, Johnson LR, Martin P et al. What causes canine sino-nasal aspergillosis? A molecular approach to species identification. *Vet J*. 2014; **200**: 17–21.

9. Barrs VR, van Doorn TM, Houbraken J et al. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One*. 2013; **8**: e64871.

10. Talbot JJ, Houbraken J, Frisvad JC et al. Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for animal and human fungal pathogens. *PLoS One*. 2017; **12**: e0181660.

11. Samson RA, Houbraken J, Thrane U, Frisvad JC (eds). *Food and Indoor Fungi*. Utrecht: Centraalbureau voor Schimmelcultures, 2010.

12. White TJ, Bruns T, Lee SJWT. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols. A Guide to Methods and Applications*. New York: Academic Press, 1990: 315–322.

13. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol*. 1995; **61**: 1323–1330.

14. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids*. 1999; **41**: 95–98.

15. Raja HA, Miller AN, Pearce CJ, Oberlies NH. Fungal identification using molecular tools: a primer for the natural products research community. *J Nat Prod*. 2017; **80**: 756–770.

16. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*. 2013; **30**: 2725–2729.

17. Pospísil K, Straka V, Otcenasek M et al. Mycotic ulcerative aortitis after replacement of the aortic valve caused by the fungus *Aspergillus restrictus*. *Vnitř Lék.* 1984; **30**: 292–297.

18. Mencl K, Otčenàšek M, Špaček J, Řehulová E. *Aspergillus restrictus* and *Candida parapsilosis* - agents of endocarditis after heart valve replacements. *Mykosen*. 1985; **28**: 127–133.

19. Silvanose CD, Bailey TA, Di Somma A. Susceptibility of fungi isolated from the respiratory tract of falcons to amphotericin B, itraconazole and voriconazole. *Vet Rec*. 2006; **159**: 282–284.

20. Burco JD, Massey G, Byrne BA et al. Monitoring of fungal loads in seabird rehabilitation centers with comparisons to natural seabird environments in Northern California. *J Zoo Wildl Med*. 2014; **45**: 29–40.

21. Burco JD, Etienne KA, Massey JG, Ziccardi MH, Balajee SA. Molecular sub-typing suggests that the environment of rehabilitation centers may be a potential source of *Aspergillus fumigatus* infecting rehabilitating seabirds. *Med Mycol*. 2012; **50**: 91–98.

22. Reif JS. Animal sentinels for environmental and public health. *Public Health Rep*. 2011; **126**: 50–57.

4.4 Conclusions

This chapter demonstrates that similar to humans and dogs, *A. fumigatus* is the most common cause of aspergillosis in captive birds in Australia (Balajee et al., 2009, Alastruey-Izquierdo et al., 2013, Negri et al., 2014, Sabino et al., 2014, Talbot et al., 2014), contrasting with Australian cats with sino-orbital aspergillosis, where cryptic *Aspergillus* species are most commonly identified (Kano et al., 2008, Barrs et al., 2012, Kano et al., 2013, Barrs et al., 2013, Barrs et al., 2014, Barrs and Talbot, 2014). No members of the *Aspergillus viridinutans* species complex (AVSC) were isolated in the study. However, another *Aspergillus* section *Fumigati* species, *A. restrictus*, which was identified is a previously unknown avian pathogen.

Avian aspergillosis is thought to arise when an immunocompromised bird encounters a high environmental load of *Aspergillus* spores, most commonly associated with poor husbandry. Thus, finding *A. fumigatus* is the most commonly isolated species is not surprising as this is the most readily isolated fungus in the environment (Sabino et al., 2014). This is similar to the findings of Chapter 3, where *A. fumigatus* was the most common *Aspergillus* species recovered from Australian domestic environments. Avian aspergillosis has predominantly been reported in captive birds, including wildlife undergoing rehabilitation,

production and sporting birds (e.g. racing pigeons, falconry) (Balseiro et al., 2005, Beernaert et al., 2010, Burco et al., 2012, Neumann, 2016). The pathogenesis involves increased environmental burdens of fungal spores due to facility location (region, climate), and / or poor husbandry (poor ventilation, poor hygiene and sanitation, animal stocking densities, vegetation build-up, substrate removal and contaminated feed), plus host factors, including impaired immunity (disease or therapeutic induced) and/ or stress (Balseiro et al., 2005, Beernaert et al., 2010, Burco et al., 2012).

Including zoos allowed the collection of isolates from a diverse range of avian species, including ducks, estrildid finches, owls, parrots, orioles, penguins, pheasants, poultry, ground-dwelling pedianomids, honeyeaters, songbirds, starlings and wading ibis. Importantly, the reporting of confirmed disease in several critically endangered native Australian birds highlights the importance of strategies to reduce the risk of aspergillosis in captive breeding recovery programs for these species.

Aspergillosis cases included in this study were mostly invasive, fatal disease with most cases diagnosed post-mortem. Given avian infection is usually associated with high environmental burdens of fungal spores, determining the species of *Aspergillus* causing infection may not only be important for managing patients diagnosed ante-mortem. Additionally, birds can act as sentinels for human disease so knowing the pathogens causing infection can be vital in disease surveillance (Reif, 2011). Finally, for treating cases, identifying the pathogen can be crucial to selecting the most appropriate treatment (Balajee et al., 2009, Zbinden et al., 2012, Alastruey-Izquierdo et al., 2013, Negri et al., 2014, Gautier et al., 2016).

This investigation identified a high proportion of clinical *A. fumigatus* sens. str. isolates, and a selection of these will be examined for azole resistance in Chapter 6.

4.5 References

ALASTRUEY-IZQUIERDO, A., MELLADO, E., PELÁEZ, T., PEMÁN, J., ZAPICO, S., ALVAREZ, M., RODRÍGUEZ-TUDELA, J. L., CUENCA-ESTRELLA, M. & AND FILPOP

STUDY GROUP 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrobial Agents and Chemotherapy,* 57**,** 3380-3387.

BALAJEE, S. A., HOUBRAKEN, J., VERWEIJ, P. E., HONG, S. B., YAGHUCHI, T., VARGA, J. & SAMSON, R. A. 2007. *Aspergillus* species identification in the clinical setting. *Studies in Mycology,* 59**,** 39-46.

BALAJEE, S. A., KANO, R., BADDLEY, J. W., MOSER, S. A., MARR, K. A., ALEXANDER, B. D., ANDES, D., KONTOYIANNIS, D. P., PERRONE, G., PETERSON, S., BRANDT, M. E., PAPPAS, P. G. & CHILLER, T. 2009. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *Journal of Clinical Microbiology,* 47**,** 3138-3141.

BALSEIRO, A., ESPI, A., MARQUEZ, I., PEREZ, V., FERRERAS, M. C., MARIN, J. F. & PRIETO, J. M. 2005. Pathological features in marine birds affected by the prestige's oil spill in the north of Spain. *Journal of Wildlife Diseases,* 41**,** 371-8.

BARRS, V. R., BEATTY, J. A., DHAND, N. K., TALBOT, J. J., BELL, E., ABRAHAM, L. A., CHAPMAN, P., BENNETT, S., VAN DOORN, T. M. & MAKARA, M. 2014. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. *The Veterinary Journal,* 201**,** 215-222.

BARRS, V. R., HALLIDAY, C., MARTIN, P., WILSON, B., KROCKENBERGER, M., GUNEW, M., BENNETT, S., KOEHLMEYER, E., THOMPSON, A., FLIEGNER, R., HOCKING, A., SLEIMAN, S., O'BRIEN, C. & BEATTY, J. A. 2012. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *The Veterinary Journal,* 191**,** 58-64.

BARRS, V. R. & TALBOT, J. J. 2014. Feline aspergillosis. *Veterinary Clinics of North America: Small Animal Practice,* 44**,** 51-73.

BARRS, V. R., VAN DOORN, T. M., HOUBRAKEN, J., KIDD, S. E., MARTIN, P., PINHEIRO, M. D., RICHARDSON, M., VARGA, J. & SAMSON, R. A. 2013. *Aspergillus*

felis sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One,* 8**,** e64871.

BEERNAERT, L. A., PASMANS, F., VAN WAEYENBERGHE, L., DORRESTEIN, G. M., VERSTAPPEN, F., VERCAMMEN, F., HAESEBROUCK, F. & MARTEL, A. 2009. Avian *Aspergillus fumigatus* strains resistant to both itraconazole and voriconazole. *Antimicrobial Agents and Chemotherapy,* 53**,** 2199-201.

BEERNAERT, L. A., PASMANS, F., VAN WAEYENBERGHE, L., HAESEBROUCK, F. & MARTEL, A. 2010. *Aspergillus* infections in birds: a review. *Avian Pathology,* 39**,** 325-31.

BURCO, J. D., ETIENNE, K. A., MASSEY, J. G., ZICCARDI, M. H. & BALAJEE, S. A. 2012. Molecular sub-typing suggests that the environment of rehabilitation centers may be a potential source of *Aspergillus fumigatus* infecting rehabilitating seabirds. *Medical Mycology,* 50**,** 91-8.

DE OCA, V. M., VALDES, S. E., SEGUNDO, C., GOMEZ, G. G., RAMIREZ, J. & CERVANTES, R. A. 2017. Aspergillosis, a Natural Infection in Poultry: Mycological and Molecular Characterization and Determination of Gliotoxin in Aspergillus fumigatus Isolates. *Avian Diseases,* 61**,** 77-82.

GAUTIER, M., NORMAND, A. C. & RANQUE, S. 2016. Previously unknown species of *Aspergillus*. *Clinical Microbiology and Infection,* S1198-743X**,** 30140- 30149.

GLASS, N. L. & DONALDSON, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology,* 61**,** 1323-1330.

KANO, R., ITAMOTO, K., OKUDA, M., INOKUMA, H., HASEGAWA, A. & BALAJEE, S. A. 2008. Isolation of *Aspergillus udagawae* from a fatal case of feline orbital aspergillosis. *Mycoses,* 51**,** 360-361.

KANO, R., SHIBAHASHI, A., FUJINO, Y., SAKAI, H., MORI, T., TSUJIMOTO, H., YANAI, T. & HASEGAWA, A. 2013. Two cases of feline orbital aspergillosis due to

Aspergillus udagawae and *A. viridinutans*. *The Journal of Veterinary Medical Science,* 75**,** 7-10.

KATZ, M. E., LOVE, S. C. J., GILL, H. S. & CHEETHAM, B. F. 1996. Development of a method for the identification, using the polymerace chain reaction, of *Aspergillus fumigatus* isolated from ostriches. *Australian Veterinary Journal,* 74**,** 50-54.

LAIR-FULLERINGER, S., GUILLOT, J., DESTERKE, C., SEGUIN, D., WARIN, S., BEZILLE, A., CHERMETTE, R. & BRETAGNE, S. 2003. Differentiation between Isolates of *Aspergillus fumigatus* from Breeding Turkeys and Their Environment by Genotyping with Microsatellite Markers. *Journal of Clinical Microbiology,* 41**,** 1798-1800.

LANÇA, A., ALMEIDA, I., MARTINS, H. M., BERNARDO, F., GUERRA, M., INÁCIO, J. & MARTINS, M. L. 2010. Polyphasic characterization of Aspergillus fumigatus strains causing infection in parrots and dolphins. *In:* MENDEZ-VILAS A (ed.) *Microorganisms in Industry and Environment – From scientific and industrial research to consumer products.* Singapore: World Scientific Publishing.

LI, H., ZHU, R., SHE, R., ZHANG, C., SHI, R., LI, W., DU, F., WU, Q., HU, F., ZHANG, Y., SOOMRO, M. H. & ZHENG, C. 2015. Case Report Associated with aspergillosis and hepatitis E virus coinfection in Himalayan Griffons. *BioMed Research International,* 2015**,** 287315.

NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION. 2016. *Basic Local Alignment Search Tool (BLAST): Standard Nucleotide BLAST* [Online]. Available: http://blast.ncbi.nlm.nih.gov/Blast.cgi [Accessed 15 July 2016].

NEGRI, C. E., GONÇALVES, S. S., XAFRANSKI, H., BERGAMASCO, M. D., AQUINO, V. R., CASTRO, P. T. & COLOMBO, A. L. 2014. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. *Journal of Clinical Microbiology,* 52**,** 3633-3640.

NEUMANN, F. E. 2016. Aspergillosis in domesticated birds. *Journal of Comparative Pathology,* 155**,** 102-4.

OLIAS, P., GRUBER, A. D., HAFEZ, H. M., LIERZ, M., SLESIONA, S., BROCK, M. & JACOBSEN, I. D. 2011. Molecular epidemiology and virulence assessment of *Aspergillus fumigatus* isolates from white stork chicks and their environment. *Veterinary Microbiology,* 148**,** 348-55.

REIF, J. S. 2011. Animal sentinels for environmental and public health. *Public Health Reports,* 126**,** 50-57.

SABINO, R., VERÍSSIMO, C., PARADA, H., BRANDÃO, J., VIEGAS, C., CAROLINO, E., CLEMONS, K. V. & STEVENS, D. A. 2014. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. *Medical Mycology,* 52**,** 519-529.

TALBOT, J. J., JOHNSON, L. R., MARTIN, P., BEATTY, J. A., SUTTON, D. A., BILLEN, F., HALLIDAY, C. L., GIBSON, J. S., KIDD, S., STEINER, J. M., UJVARI, B. & BARRS, V. R. 2014. What causes canine sino-nasal aspergillosis? A molecular approach to species identification. *The Veterinary Journal,* 200**,** 17-21.

THIERRY, S., WANG, D., ARNE, P., DEVILLE, M., DE BRUIN, B., NIEGUITSILA, A., POURCEL, C., LAROUCAU, K., CHERMETTE, R., HUANG, W., BOTTEREL, F. & GUILLOT, J. 2010. Multiple-locus variable-number tandem repeat analysis for molecular typing of *Aspergillus fumigatus*. *BMC Microbiology,* 10**,** http://www.biomedcentral.com/1471-2180/10/315.

VAN WAEYENBERGHE, L., PASMANS, F., BEERNAERT, L. A., HAESEBROUCK, F., VERCAMMEN, F., VERSTAPPEN, F., DORRESTEIN, G. M., KLAASSEN, C. H. & MARTEL, A. 2011. Microsatellite typing of avian clinical and environmental isolates of *Aspergillus fumigatus*. *Avian Pathology,* 40**,** 73-7.

WANG, D. Y., GRICOURT, M., ARNE, P., THIERRY, S., SEGUIN, D., CHERMETTE, R., HUANG, W. Y., DANNAOUI, E., BOTTEREL, F. & GUILLOT, J. 2014. Mutations in the *Cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* isolated from avian farms in France and China. *Poultry Science,* 93**,** 12-5.

WHITE, T. J., BRUNS, T. & LEE, S. J. W. T. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* M. A. INNIS, D. H. G., J. J. SNINSKY & T. J. WHITE, (ed.) *PCR Protocols. A Guide to Methods and Applications.* New York: Academic Press.

ZBINDEN, A., IMHOF, A., WILHELM, M. J., RUSCHITZKA, F., WILD, P., BLOEMBERG, G. V. & MUELLER, N. J. 2012. Fatal outcome after heart transplantation caused by *Aspergillus lentulus*. *Transplant Infectious Disease,* 14**,** E60-E63.

Chapter 5. Azole resistance amongst canine and feline sino-nasal aspergillosis isolates

5.1 Introduction

Aspergillus fumigatus is the most common cause of sino-nasal aspergillosis (SNA) in dogs. As with human aspergillosis, azole therapeutics are also the mainstay for treating veterinary aspergillosis. Surgical debridement of fungal plaques and application of topical azole antifungal drugs (clotrimazole or enilconazole) is the recommended treatment for canine SNA, and where topical treatment is not possible, systemic antifungal azole drugs are recommended (e.g. itraconazole) (Zonderland et al., 2002, Claeys et al., 2006, Billen et al., 2010, Stengel, 2017). Multiple treatments are often required and a mortality rate up to 13% is reported in dogs following one treatment with fungal plaque debridement and topical application of either clotrimazole or enilconazole (Sharman et al., 2010). Additionally, *in vitro* antifungal susceptibility testing data for isolates in the literature are limited (Lanthier and Chalifoux, 1991, Sharp et al., 1993). This chapter investigates if azole- resistant *A. fumigatus* (ARAf) are a cause of treatment failure in dogs, as in human patients throughout the world. This was investigated through microbroth dilution antifungal susceptibility testing and *cyp51A* sequencing.

5.2 Premise

Antifungal susceptibility is often tested using *in vitro* microbroth dilution to determine the susceptibility of *A. fumigatus* to antifungal drugs. It is employed for epidemiological research as well as management of human clinical cases where results can be used to predict the *in vivo* response to therapy and recognise resistant isolates. Epidemiological cut-off values (ECVs) and EUCAST proposed breakpoints have been reported for most antifungal therapies used to treat aspergillosis in people (Rodriguez-Tudela et al., 2008, Verweij et al., 2009, Pfaller et al., 2009, Espinell-Ingroff et al., 2010, Espinel-Ingroff et al., 2013, Wiederhold et al., 2016, Clinical Laboartory Standards Institute (CLSI), 2017, EUCAST, 2017, Clinical and Laboratory Standards Institute (CLSI), 2018). These are used to interpret minimum inhibitory concentration (MIC) values by distinguishing between wild-type (susceptible) and resistant phenotypes.

This chapter builds on an investigation I commenced during my undergraduate BSc(vet)(hons) degree where I identified 88 isolates of *A. fumigatus* from dogs with clinically confirmed cases of SNA in Australia, the USA and Belgium (Talbot et al., 2014) and learnt antifungal susceptibility testing techniques based on CLSI methodology (Rex et al., 2008). This chapter further investigates the *in vitro* antifungal susceptibility profiles of a selection of these isolates from 46 dogs (29 from Australia, 7 from Belgium, 10 from the USA) plus isolates from four Australian cats also with sino-nasal aspergillosis against azole antifungal therapeutics, including clotrimazole and enilconazole. This was achieved via the CLSI microbroth dilution method M38-A2 standard method (for clotrimazole and enilconazole susceptibility), (Rex et al., 2008) and the colorimetric microbroth dilution in house commercial testing kit Sensititre™ YeastOne™ (Thermo Scientific Trek Diagnostic Systems, USA) to test itraconazole, voriconazole, posaconazole, ketoconazole, fluconazole, amphotericin B, caspofungin and 5-flucytosine C. I identified one canine isolate from Australia with *in vitro* resistance to itraconazole and ketoconazole, and elevated MICs for voriconazole and posaconazole.

This chapter also examines the *cyp51A* gene for isolates with *in vitro* resistance. Because mutations in the *cyp51A* gene have been identified as the most common cause of azole resistance in human and environmental *A. fumigatus* isolates, I performed *cyp51A* sequencing to determine if mutations known to cause resistance in human and environmental *A. fumigatus* isolates were also present amongst veterinary ARAf isolates using previously published primers (Chen et al., 2005).

While clotrimazole and enilconazole are the topical treatment of choice for dogs and cats with SNA (Goodall et al., 1984, Zonderland et al., 2002, Tomsa et al., 2003, Claeys et al., 2006, Furrow and Groman, 2009, Billen et al., 2010, Barrs et al., 2012, Tamborini et al., 2016, Stengel, 2017), they are not used for treatment of aspergillosis in people, and no ECVs or proposed breakpoints have been established for these against *A. fumigatus*. Antifungal susceptibility reports for these drugs in the canine SNA literature are largely lacking. Therefore, this chapter investigates the prevalence of ARAf in canine and feline SNA isolates and provides the largest dataset of MIC ranges of clotrimazole and enilconazole against canine isolates of *A. fumigatus*.

5.3 Publication

The following publication was re-formatted to suit the style of this thesis. The publication's in-text references remain in the style required by the journal (number brackets form) and the publication associated tables and references follow the main text. This research is presented in its original publication form in **Appendix 4**. A list of all other references referred to in this chapter outside of section **5.3 Publication** can be found at the chapter's end (see **5.5 References**).

Talbot JJ., Kidd SE., Martin P., Beatty JA., Barrs VR. Azole resistance in canine and feline isolates of *Aspergillus fumigatus. Comparative Immunology, Microbiology and Infectious Diseases.*2015; 42: 37-41.

Abstract

Azole resistance is an emerging cause of treatment failure in humans with aspergillosis. The aim of this study was to determine if azole resistance is emerging in *Aspergillus fumigatus* isolates from canine and feline sino-nasal aspergillosis cases. Susceptibilities of isolates collected between 1988 and 2014 from 46 dogs and 4 cats to itraconazole, posaconazole, voriconazole, fluconazole and ketoconazole were assessed using Sensititre YeastOne microdilution trays; and to enilconazole and clotrimazole, following the CLSI M38-A2 standard. For the majority of isolates MICs were high for ketoconazole, low for enilconazole and clotrimazole, and less than established epidemiological cut-off values for itraconazole, posaconazole and voriconazole. One canine isolate from 1992 had multiazole resistance and on C*yp51A* gene sequencing a mutation associated with azole resistance (F46Y) was detected. There is no evidence of emerging azole resistance among *A. fumigatus* isolates from dogs and cats and topical azole therapy should be effective against most isolates.

Introduction

Azole antifungal drugs are pillars of treatment for aspergillosis in humans and animals. Since 1997, there has been an increasing number of reports of azole resistance amongst isolates of *Aspergillus fumigatus* from human patients with both invasive and non-invasive forms of aspergillosis. Azole resistance is associated with treatment failures and increased mortality [1–3]. Resistance to itraconazole alone or multiazole or panazole resistance has been reported globally [4–7].

Fungal azole resistance can be intrinsic or acquired. Intrinsic resistance is reported for cryptic *Aspergillus* species, whereas acquired resistance is increasingly described for *A. fumigatus sensu stricto* isolates [8,9]. Resistant strains have been identified in the environment and in human patients naïve to and previously exposed to azole therapy [10]. Resistance is associated with mutations in the c*yp51A* gene which encodes lanosterol 14α-demethylase, a component of the ergosterol synthesis pathway targeted by triazole drugs [9,11]. Emergence of resistance in Europe has been linked to triazole fungicide use in agriculture causing altered gene expression [12].

Clinical antifungal resistance is defined as persistence of infection despite treatment with an antifungal with proven *in vitro* activity against the infecting fungus [13]. *In vitro* resistance is difficult to define as although standardised methods of anti-fungal susceptibility testing have been developed [14–16], clinical breakpoints based on minimum inhibitory concentrations (MICs), pharmacological parameters, animal data and clinical outcomes, have not yet been established for *Aspergillus* species. Instead, epidemiological cut-off values (ECVs) of wild-type *A. fumigatus* isolates are used to evaluate clinical isolate minimum inhibitory concentrations (MICs). Correlation between *in vitro* and clinical outcomes is reported from invasive and non-invasive human aspergillosis cases [1–3].

Canine and feline sino-nasal aspergillosis (SNA) is most commonly caused by members of *Aspergillus* section *Fumigati*. Canine SNA is usually caused by *A. fumigatus* [17] while feline SNA is most commonly caused by *A. fumigatus* and *Aspergillus niger* [18]. These infections present therapeutic challenges for veterinarians, and involve the use of endosurgical procedures and topical and/or systemic antifungal azole administration. Topical clotrimazole (CLO) or enilconazole (ENL) are the mainstays of therapy, although there are limited *in vitro* studies to support the use of these drugs [19,20]. Response rates to treatment vary [18,21,22]. Whether emerging azole resistance contributes to treatment failures is unknown. To date no studies have systematically investigated the prevalence of azole resistance amongst *A. fumigatus* isolates from dogs and cats. To determine whether azole resistance is emerging amongst *A. fumigatus* isolates from dogs and cats susceptibilities to a panel of antifungal drugs were assessed in a retrospective surveillance study.

Materials and Methods

Isolates

Fifty archived isolates of *A. fumigatus* from dogs (*n* = 46) and cats (*n* = 4) with confirmed SNA were retrieved from the fungal culture biobank of the Small Animal Infectious Diseases Clinical Research Group at the University of Sydney [17,21,23]. Isolates originated from Australia (*n* = 33), United States of America (*n* = 10) and Belgium (*n* = 7). Collection dates ranged from 1988 to 2013 inclusive [1988 (*n* = 1); 1992 (*n* = 1); 1994 (*n* = 1); 2000 (*n* = 1); 2004 (*n* = 1); 2005 (*n* = 1); 2006 (*n* = 1); 2007 (*n* = 5); 2008 (*n* = 7); 2009 (*n* = 5); 2010 (*n* = 6); 2011 (*n* = 3); 2012 (*n* = 14); 2013 (*n* = 3)].

Antifungal susceptibility testing

Isolates were sub-cultured onto malt extract agar and grown in the dark at 25 °C until sporulation occurred (7–14 days), then inoculum was prepared according to the Clinical Laboratory Standards Institute (CLSI) M38-A2 standard for filamentous fungi [16].

Susceptibility (*n* = 50) to ENL and CLO (BOVA Compounding Chemist, St Marys, Australia) was assessed following the recommendations for drug preparation, dilution, inoculum preparation, incubation and endpoint reading stipulated by the CLSI M38-A2 standard [16]. Final concentrations tested were two fold dilutions within the range 0.03–16 μg/mL for both compounds. The concentrations selected were based on *in vitro* susceptibilities of *A. fumigatus* to other azole drugs [16,24,25] and are lower than concentrations of 1–2% (10–20 mg/mL) used in topical preparations of clotrimazole and enilconazole for treatment of canine and feline SNA [19–21,24,25]. Quality control strains used as a standard to assess repeatability and accuracy of testing procedures were reference strains of *Candida parapsilosis* (ATCC 22019) and *A. fumigatus (*ATCC MYA-3626), according to the CLSI M38-A2 standard.

Susceptibility to amphotericin B (AMB), itraconazole (ITZ), ketoconazole (KCZ), voriconazole (VCZ) (range 0.008–16 μg/mL for all four compounds), posaconazole (POS) (range 0.008–8 μg/mL), fluconazole (FLU) (0.12–256 μg/mL) and 5-flucytosine (5-FC) (0.03–64 μg/mL) was assessed using Sensititre YeastOne YO8 microdilution trays according to the manufacturer's instructions. Susceptibility to caspofungin (CSP) (range 0.008–16 μg/mL) was assessed using the CLSI M38-A2 standard [16]. For those drugs for which pharmacokinetic data are available in dogs and/or cats including itraconazole, fluconazole, ketoconazole and

posaconazole, reported C_{MAX} in serum or plasma after therapeutic dosing were within the range of drug concentrations tested in this study [26–31]. *C. parapsilosis* (ATCC 22019) was used as the quality control strain (Trek Diagnostic Systems, Thermo Fisher Scientific, Scoresby, Australia).

For all isolates and both tray types, 10 μL from the growth control well was spread onto a Sabouraud's agar plate immediately after plate inoculation as a purity and inoculum density check. All trays were incubated at 35 °C for 72 h ± 2 h in an ambient air incubator. Endpoints for all drugs were determined after 72 h incubation. For CLO and ENL the endpoint was the lowest two-fold dilution revealing 100% growth inhibition (MIC). For AMB, ITZ, POS, VCZ, FLU, KCZ and 5-FC, the endpoints were as per the manufacturer's instructions (Trek Diagnostic Systems, Thermo Fisher Scientific, Scoresby, Australia). For CSP endpoints were read as MECs at the lowest dilution of round compact hyphal forms as per CLSI M38A2 [16]. Geometric mean, MIC_{50} and MIC_{90} values were calculated using Windows Excel (Microsoft Office, 2007, Redmond, United States of America).

In vitro azole resistance was defined as the absence of endpoint antifungal activity in the range of drug concentrations tested, except for FLU and 5-FC, as most filamentous fungi have intrinsic resistance to these drugs [16]. Multiazole resistance was defined as resistance to more than one antifungal azole. Decreased susceptibility was defined as MICs greater than established ECVs for wild-type *A. fumigatus*, established using CLSI standards [16].

Cyp51A gene sequencing

DNA was extracted from those isolates with possible ITZ resistance (*i.e.* MICs > 1 μg/mL) using the Roche High Pure PCR Template Preparation Kit (Castle Hill, Australia), with an additional bead beating step [32]. The coding region of *cyp51A* was sequenced as described previously [11]. Sequences were aligned with *cyp51A A. fumigatus* reference strain, GenBank reference AF338659 [33] and sequence from a reference laboratory isolate with a L98H *cyp51A* resistance mutation (obtained from National Mycology Reference Centre, SA Pathology, Adelaide, South Australia, 2014).

Results

Antifungal susceptibility results for all 50 *A. fumigatus* isolates from clinical specimens from dogs and cats with sino-nasal aspergillosis are listed in Table 1 and compared to established epidemiological cut-off values (ECVs) where these have been established. All isolates had high MICs for KCZ. As expected, there was no observed activity of FLU or 5-FC against any isolate. Forty-nine of the 50 isolates had low MICs for ITZ, VCZ, POS and low MECs for CSP. Seven isolates had MICs higher than the ECV for AMB. The *C. parapsilosis* (ATCC 22019) quality control strain had MIC values within the manufacturer's defined range defined within the YeastOne YO8 product insert.

The majority of *A. fumigatus* isolates had low MICs for ENL and CLO. The *A. fumigatus (*ATCC MYA-3626) quality control strain had a MIC value of 1 μg/mL for CLO and 0.25 μg/mL for ENL. The *C. parapsilosis* (ATCC 22019) quality control strain had a MIC value of 0.125 μg/mL for CLO and 0.25 μg/mL for ENL.

There was no activity of ITZ and KCZ against one Australian canine *A. fumigatus* isolate collected in 1992. The same isolate also showed high MICs of VCZ and POS (Table 2). *Cyp51A* sequencing revealed non-synonymous mutations at nucleotide positions 137 (F46Y), 267 (G89E), 1279 (E427K) and a synonymous mutation at nucleotide 1362 (C454).

Discussion

In this study azole resistance was rare amongst *A. fumigatus* isolates from dogs and cats. ITZ resistance in *Aspergillus* species was first reported in 1997 from Californian isolates sampled from human patients in the late 1980s [34]. Since the late 1990s, there has been an increase in the number of reports of and surveillance programs for azole resistance in human *A. fumigatus* isolates [35]. Azole resistance in environmental and human clinical isolates has been reported in Europe, Asia, North and South America, Australia and the Middle East [2,4,5,7,36]. Emergence of resistance in Europe has been linked to triazole fungicide use on crops causing altered gene expression and this has prompted a number of surveillance programs that have continued to identify azole resistance in the region, predominantly amongst isolates of *A. fumigatus* [10,12].

The only isolate in this study to demonstrate *in vitro* azole resistance was collected from a dog in Australia in 1992. This isolate exhibited multiazole resistance, with no observed activity of

ITZ/KCZ in the range tested (MIC > 16 μg/mL) (Table 2). Itraconazole MIC values >8 μg/mL in *A. fumigatus* isolates from humans with invasive aspergillosis have been associated with clinical resistance [14,34]. Correlations between *in vitro* and clinical activity of KCZ and *A. fumigatus* isolates from humans have also been reported [37]. A limitation of the current study was the lack of detailed treatment histories for affected dogs and cats.

This is the first study to systematically confirm *in vitro* susceptibility of canine and feline isolates of *A. fumigatus* to ENL and CLO using standardized methods. Pharmaceutical company MIC values for ENL against 77 *Aspergillus* spp. canine isolates were reported by a pharmaceutical company previously and were ≤1 μg/mL in 62% of these (range ≤0.1– 10 μg/mL) [19]. However, isolates were not identified to species level, and the method for antifungal susceptibility testing was not described. Another study on the use of ENL to treat canine SNA reported a MIC value for *A. fumigatus* to ENL of 1 μg/mL [20]. However, the number of isolates tested and the method of testing was not reported.

We found that canine and feline isolates of *A. fumigatus* are susceptible to ENL and CLO. *A. fumigatus* isolates had MIC values for CLO between 0.25 and 4 μg/mL and for ENL between 0.06 and 2 μg/mL. Therefore, assuming that *in vitro* MICs correlate with clinical response, currently used concentrations (10 mg/mL) of topical azole therapy should be effective against *A. fumigatus* isolates causing SNA in dogs and cats, and azole resistance is unlikely to be a cause of treatment failure at this time. One percent solutions of CLO and ENL are standard for the treatment of canine SNA, with approximately 50 mL of 10 mg/mL solution infused into the sino-nasal cavities of large breed dogs to ensure that high concentrations of the drug contact fungal plaques [24,25]. Additional treatment with at least 20 g of CLO 10 mg/g cream has also been used following infusion therapy [24]. CLO infusions have also been used in cats with SNA [18].

A recent study by the authors to determine the molecular identification of canine SNA isolates confirmed the most common aetiological agent as *A. fumigatus* (88/91 isolates), thus different aetiological agents are not the cause of the varied clinical response to treatment reported in dogs [17]. CLO and ENL were found to have *in vitro* activity against all isolates in this study. A likely impediment to disease resolution is the inability of azoles to adequately penetrate into mucosal fungal plaques. Endoscopic debridement of all visible plaques is considered essential to achieve disease resolution in both dogs and cats with SNA [18,38]. In human fungal

rhinosinusitis due to *A. fumigatus*, fungal balls are endosurgically removed and usually no topical antifungal therapy is applied or required to achieve therapeutic success [39,40]. Another possible cause of treatment failure in canine and feline SNA is a lack of adequate drug distribution to the affected areas of the nasal cavity and frontal sinus when applying topical preparations of azoles [38,41,42].

Topical azole therapy is contraindicated in the presence of cribriform plate breaches in dogs and cats with SNA due to the potential for chemical meningoencephalitis. In these cases systemic antifungal therapy is offered in addition to topical fungal plaque debridement. We recommend that *in vitro* antifungal susceptibility testing be performed to determine resistant phenotypes. The seven isolates with high MICs for AMB (>2 μg/mL) in this study highlight the importance of performing *in vitro* susceptibility in cases where systemic therapy is being considered. In human studies, MIC values >2 μg/mL have been associated with treatment failure, while values <2 μg/mL have been associated with treatment success in patients with invasive *A. fumigatus* infections [43]. The significance of these MIC values has not yet been determined dogs and cats with sino-nasal aspergillosis.

High KCZ MIC results ($\geq MIC_{90}$ 8 μg/mL) were present in 38% (19/50) of *A. fumigatu*s isolates in this study, suggesting that KCZ is unsuitable for treatment of canine or feline SNA. These MICs are similar to those reported for human clinical *A. fumigatus* isolates [44,45].

Combinations of topical azoles and systemic antifungals have also been used to treat canine and feline SNA. Previously KCZ and topical ENL were used for canine SNA treatment [19]. More recently combination therapy with oral ITZ and topical therapy has been employed [46]. Feline SNA has also been treated with a combination of systemic and topical intranasal infusions or topical intranasal infusions only. Therapeutic success of systemic (ITZ or POS ± AMB), topical (CLO) and combined antifungal therapies has been reported in 14 cases of feline SNA, with a success rate of 79% [18].

Epidemiological cut-off values (ECVs) for wild type *A. fumigatus* isolates to AMB, ITZ, POS, VCZ and CSP have been established using CLSI standards for broth microdilution testing. These ECVs may be useful for identifying resistant phenotypes with non-wild type MICs. For AMB 3988 wild type *A. fumigatus* isolates were tested and 95% had MICs of 2 μg/mL [47]. In the current study, 86% (n = 43) of the *A. fumigatus* isolates were found to have

MICs ≤ 2 μg/mL. The remaining 14% of isolates (*n* = 7) had higher MIC values (4 μg/mL). Of 1684–2815 wild type, *A. fumigatus* isolates tested for susceptibility to ITZ, POS and VCZ, 98.8% had MIC values of 1 μg/mL for ITZ; 99.2% had MICs of 0.5 μg/mL for POS; and 97.7% had MICs of 1 μg/mL for VCZ [48]. In the current study all isolates had MICs for POS ≤ 0.5 μg/mL and only one isolate had ITZ MIC > 1 μg/mL (16 μg/mL). The same isolate and one USA isolate from 2008 had VCZ MICs > 1 μg/mL (4 μg/mL and 2 μg/mL, respectively). Of 1691 wild type *A. fumigatus* isolates tested for susceptibility to CSP, 99% had MECs of 1 μg/mL [49]. In the current study all isolates had MECs for CSP <1 μg/mL. These results imply that compared to the ECVs for wild type *A. fumigatus* isolates, resistant phenotypes were rare amongst this cohort of isolates, although resistance for AMB (*n* = 7), ITZ (*n* = 1) and VCZ (*n* = 2) was observed.

The most common *cyp51A* mutations associated with azole resistance in *A. fumigatus* are a SNP (L98H) in combination with a 34 bp tandem repeat in the gene promoter region (TR_{34}/L98H) or a 46 bp tandem repeat in the same region and two separate SNPs (TR_{46}/Y156F/T289A) [4]. In this study the *cyp51A* amino acid substitutions found in the 1992 azole resistant canine isolate mostly occurred in regions outside the coding region and are therefore unlikely to contribute to resistance [50,51]. However, the F46Y mutation we detected, has been previously associated with reduced susceptibility to triazoles [7]. Azole resistant isolates lacking mutations in the *cyp51A* gene have been previously reported [50]. Other proposed resistance mechanisms include upregulation of efflux pumps, expression levels of transporters and multidrug resistance proteins or other undiscovered mechanisms [50].

Conclusions

This study determined that azole resistance is not emerging in *A. fumigatus* isolates from dogs and cats with SNA. In general, MICs were similar to those reported for wild type isolates for ITZ, POS, VCZ, AMB and CSP, implying that canine and feline isolates of *A. fumigatus* are generally susceptible to these antifungals. MICs of *A. fumigatus* isolates to ENL and CLO were relatively low and concentrations currently used for topical therapy should be effective. Further studies would be useful to investigate *in vitro* versus clinical outcomes in a greater number of cases to help develop interpretive guidelines for antifungal susceptibility testing.

Acknowledgements

The authors thank Jan Bell, SA Pathology at the Women's and Children's Hospital, North Adelaide, for technical assistance.

Publication Tables

Table 1. Antifungal susceptibility results for 50 *A. fumigatus* isolates from clinical specimens from dogs and cats and comparison with epidemiological cut-off values.* MIC/MEC (µg/mL) values reflect the number of isolates within the specific cut-off value.

Drug	MIC/MEC (µg/mL) distribution among tested isolates												GM	MIC_{50}	MIC_{90}	ECV(µg/mL)
	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	>16				
AMB						1	8	34	7				1.92	2	4	2
ITZ		2	13	16	17	1						1	0.13	0.12	0.25	1
VCZ			2	3	24	16	3	1	1				0.34	0.25	0.5	1
POS	5	13	20	8	3	1							0.06	0.06	0.12	0.5
KCZ							1	2	28	15	3	1	5.09	4	8	–
CSP†	16	25	8	1									0.03	0.03	0.06	1
CLO					2	31	15	1	1				0.64	0.5	1	–
ENL			7	27	11	4		1					0.15	0.12	0.25	–

* ECV, epidemiological cut off value [46–48]; MIC, minimum inhibitory concentration; MEC, minimum effective concentration (†); AMB, amphotericin-B; ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; KCZ, ketoconazole; CSP, caspofungin; CLO, clotrimazole; ENL, enilconazole; GM, geometric mean; MIC_{50}, minimum inhibitory concentration at which ≥50% of isolates in the group were inhibited; MIC_{90}, minimum inhibitory concentration at which ≥90% of isolates in the group were inhibited.

Table 2. Antifungal susceptibility results for an Australian canine clinical isolate of *A. fumigatus* from 1992 compared to group GM, MIC_{50} and MIC_{90} results*.

Drug	MIC/MEC (μg/mL)	GM	MIC_{50}	MIC_{90}	ECV (μg/mL)
AMB	1	1.97	2	4	2
ITZ	>16	0.14	0.12	0.25	1
VCZ	4	0.37	0.25	0.5	1
POS	0.5	0.06	0.06	0.12	0.5
KCZ	>16	5.52	4	8	–
CSP†	0.03	0.03	0.06	0.03	1
CLO	4	0.39	0.5	0.5	–
ENL	2	0.15	0.125	0.5	–

* ECV, epidemiological cut-off values from [46–48]; MIC, minimum inhibitory concentration; MEC, minimum effective concentration (†); AMB, amphotericin-B; ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; KCZ, ketoconazole; CSP, caspofungin; CLO, clotrimazole; ENL, enilconazole.

Publication References

[1] P.R. Burgel, M.T. Baixench, M. Amsellem, et al., High prevalence of azole-resistant *Aspergillus fumigatus* in adults with cystic fibrosis exposed to itraconazole, Antimicrob. Agents. Chemother. 56 (2011) 869–874.

[2] I. Hadrich, F. Makni, S. Neji, et al., Amphotericin B in vitro resistance is associated with fatal *Aspergillus flavus* infection, Med. Mycol. 50 (2012) 829–834.

[3] J.W. van der Linden, E. Snelders, G.A. Kampinga, et al., Clinical implications of azole-resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009, Emerg. Infect. Dis. 17 (2011) 1846–1854.

[4] D.W. Denning, D.S. Perlin, Azole resistance in *Aspergillus*: a growing public health menace, Future Microbiol. 6 (2011) 1229–1232.

[5] C.D. Pham, E. Reiss, F. Hagen, et al., Passive surveillance for azole-resistant *Aspergillus fumigatus*, United States, 2011–2013, Emerg. Infect. Dis. 20 (2014) 1498–1503.

[6] P.E. Verweij, S.J. Howard, W.J. Melchers, et al., Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints, Drug Resist. Updat. 12 (2009) 141–147.

[7] S.E. Kidd, E. Goeman, J.F. Meis, et al., Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia, Mycoses 58 (2015) 350–355.

[8] S.J. Howard, Multi-resistant aspergillosis due to cryptic species, Mycopathologia 178 (2014) 435–439.

[9] J.W. van der Linden, A. Warris, P.E. Verweij, *Aspergillus* species intrinsically resistant to antifungal agents, Med. Mycol. 49 (S1) (2011) S82–S89.

[10] S.J. Howard, M.C. Arendrup, Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection, Med. Mycol. 49 (S1) (2011) S90–S95.

[11] J. Chen, H. Li, R. Li, et al., Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma, Antimicrob. Agents. Chemother. 55 (2005) 31–37.

[12] P.E. Verweij, G.H. Kema, B. Zwaan, et al., Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould *Aspergillus fumigatus*, Pest. Manage. Sci. 69 (2012) 165–170.

[13] Z.A. Kanafani, J.R. Perfect, Resistance to antifungal agents: mechanisms and clinical impact, Antimicrob. Resist. 46 (2008) 120–128.

[14] D.W. Denning, S.A. Radford, K.L. Oakley, et al., Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatus* infection, J. Antimicrob. Chemother. 40 (1997) 401–414.

[15] C. Lass-Flörl, M. Cuenca-Estrella, D.W. Denning, Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology, Med. Mycol. 44 (2006) S319–S325.

[16] J.H. Rex, B.D. Alexander, D. Andes, et al., Reference method for broth dilution antifungal susceptibility testing of filamentous fungi Approved Standard M38-A2, vol. 28, second ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2008, pp. 1–35 (no. 16).

[17] J.J. Talbot, P. Martin, L. Johnson, et al., What causes sino-nasal aspergillosis in dogs? A molecular approach to species identification, Vet. J. 200 (2014) 17–21.

[18] V.R. Barrs, J.J. Talbot, Feline aspergillosis, Vet. Clin. North Am. Small Anim. Pract. 44 (2014) 51–73.

[19] N.J.H. Sharp, M. Sullivan, C.E. Harvey, et al., Treatment of canine nasal aspergillosis with enilconazole, J. Vet. Intern. Med. 7 (1993) 40–43.

[20] T. Lanthier, A. Chalifoux, Enilconazole as an adjunct to the treatment of 4 cases of canine nasal aspergillosis, Can. Vet. J. 32 (1991) 110–112.

[21] V.R. Barrs, C. Halliday, P. Martin, et al., Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes, Vet. J. 191 (2012) 58–64.

[22] K.G. Mathews, N.J.H. Sharp, Canine nasal aspergillosis-penicilliosis, in: C.E. Green (Ed.), Infectious Diseases of the Dog and Cat, third ed., Saunders Elsevier, St Louis, MO, 2006, pp. 613–620.

[23] V.R. Barrs, T.M. van Doorn, J. Houbraken, et al., *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs, PLoS ONE 8 (2013), DOI 64810.61371/journal.pone.0064871.

[24] T.R. Sissener, N.J. Bacon, E. Friend, et al., Combined clotrimazole irrigation and depot therapy for canine nasal aspergillosis, J. Small Anim. Pract. 47 (2006) 312–315.

[25] J.L. Zonderland, C.K. Stork, J.H. Saunders, et al., Intranasal infusion of enilconazole for treatment of sinonasal aspergillosis in dogs, J. Am. Vet. Med. Assoc. 221 (2002) 1421–1425.

[26] D.M. Boothe, I. Herring, J. Calvin, et al., Itraconazole disposition after single oral and intravenous and multiple oral dosing in healthy cats, Am. J. Vet. Res. 58 (1997) 872–877.

[27] H. Van Cauteren, J. Heykants, R. De Coster, et al., Itraconazole: pharmacologic studies in animals and humans, Rev. Infect. Dis. 9 (1987) S43–S46.

[28] S.L. Vaden, M.C. Heit, E.C. Hawkins, et al., Fluconazole in cats: pharmacokinetics following intravenous and oral administration and penetration into cerebrospinal fluid, aqueous humour and pulmonary epithelial lining fluid, J. Vet. Pharmacol. Ther. 20 (1997) 181–186.

[29] M.J. Humphrey, S. Jevons, M.H. Tarbit, Pharmacokinetic evaluation of UK-49,858, a metabolically stable triazole antifungal drug, in animals and humans, Antimicrob. Agents Chemother. 28 (1985) 648–653.

[30] J.G. Baxter, C. Brass, J.J. Schentag, et al., Pharmacokinetics of ketoconazole administered intravenously to dogs and orally as tablet and solution to humans and dogs, J. Pharm. Sci. 75 (1986) 443–447.

[31] A.A. Nomeir, P. Kumari, M.J. Hilbert, et al., Pharmacokinetics of SCH 56592, a new azole broad-spectrum antifungal agent, in mice, rats, rabbits, dogs, and cynomolgus monkeys, Antimicrob. Agents Chemother. 44 (2000) 727–731.

[32] P.L. White, M.D. Perry, J. Loeffler, et al., Critical stages of extracting DNA from *Aspergillus fumigatus* in whole-blood specimens, J. Clin. Microbiol. 48 (2010) 3753–3755.

[33] E. Mellado, T.M. Diaz-Guerra, M. Cuenca-Estrella, et al., Identification of two different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in *Aspergillus fumigatus* and other *Aspergillus* species, J. Clin. Microbiol. 39 (2001) 2431–2438.

[34] D.W. Denning, K. Venkateswarlu, K.L. Oakley, et al., Itraconazole resistance in *Aspergillus fumigatus*, Antimicrob. Agents Chemother. 41 (1997) 1364–1368.

[35] S.P. Georgiadou, D.P. Kontoyiannis, The impact of azole resistance on aspergillosis guidelines, Ann. N.Y. Acad. Sci. 1272 (2012) 15–22.

[36] A. Chowdhary, S. Kathuria, J. Xu, et al., Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health, PLoS Pathog. 9 (2013), http://dx.doi.org/ 10.1371/annotation/4ffcf1da-b180-4149-834c-9c723c5dbf9b.

[37] E. Cauvenbergh, J. Symoens, Clinical experience with ketoconazole, a review, Clin. Res. Rev. 1 (1981) 195–199.

[38] M.J. Sharman, C.S. Mansfield, Sinonasal aspergillosis in dogs: a review, J. Small Anim. Pract. 53 (2012) 434–444.

[39] X. Dufour, C. Kauffman-Lacroix, J.C. Ferrie, et al., Paranasal sinus fungus ball and surgery: a review of 175 cases, Rhinology 43 (2005) 34–39.

[40] M.G. Zuniga, J.H. Turner, Treatment outcomes in acute invasive fungal rhinosinusitis, Curr. Opin. Otolaryngol. Head Neck Surg. 22 (2014) 242–248.

[41] G.M. Hayes, J.L. Demetriou, Distribution and persistence of topical clotrimazole after sinus infusion in normal canine cadavers, J. Small Anim. Pract. 53 (2012) 95–100.

[42] R. Burrow, D. McCarroll, M. Baker, et al., Frontal sinus depth at four landmarks in breeds of dog typically affected by sinonasal aspergillosis, Vet. Rec. 170 (2012), http://dx.doi.org/10.1136/vr.100041.

[43] C. Lass-Florl, G. Kofler, G. Kropshofer, et al., *In vitro* testing of susceptibility to amphotericin B is a reliable predictor of clinical outcome in invasive aspergillosis, J. Antimicrob. Chemother. 42 (1998) 497–502.

[44] A.J. Carrillo-Munoz , G. Quindós, M. Ruesga, et al., In vitro antifungal susceptibility testing of filamentous fungi with Sensititre Yeast One, Mycoses 49 (2006) 293–297.

[45] S.A. Messer, R.N. Jones, T.R. Fritsche, International Surveillance of Candida spp. and *Aspergillus* spp.: report from the SENTRY Antimicrobial Surveillance Program (2003), J. Clin. Microbiol. 44 (2006) 1782–1787.

[46] S. Claeys, J.B. Lefebvre, S. Schuller, et al., Surgical treatment of canine nasal aspergillosis by rhinotomy combined with enilconazole infusion and oral itraconazole, J. Small Anim. Pract. 47 (2006) 320–324.

[47] A. Espinel-Ingroff, M. Cuenca-Estrella, A. Fothergill, et al., Wild-Type MIC distributions and epidemiological cutoff values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document), Antimicrob. Agents Chemother. 55 (2011) 5150–5154.

[48] A. Espinel-Ingroff, D.J. Diekema, A. Fothergill, et al., Wild-Type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document), J. Clin. Microbiol. 48 (2010) 3251–3257.

[49] A. Espinel-Ingroff, A. Fothergill, J. Fuller, et al., Wild-Type MIC distributions and epidemiological cutoff values for caspofungin and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document), Antimicrob. Agents Chemother. 55 (2011) 2855–2859.

[50] E. Snelders, A. Karawajczyk, G. Schaftenaar, et al., Azole resistance profile of amino acid changes in *Aspergillus fumigatus CYP51A* based on protein homology modeling, Antimicrob. Agents Chemother. 54 (2010) 2425–2430.

[51] S.J. Howard, D. Cerar, M.J. Anderson, et al., Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure, Emerg. Infect. Dis. 15 (2009) 1068–1076.

5.4 Conclusions

This publication identified only one isolate with azole resistance amongst 53 veterinary clinical isolates of *A. fumigatus* from different global locations. The resistant isolate taken from an Australian sample from 1992 had the *cyp51A* mutations F46Y, G89E and E427K (GenBank Accession number KT366018). The mutation F46Y is associated with azole resistant and azole susceptible isolates of *A. fumigatus*. Where resistance has been observed, it has only been noted combined with other mutations, including M172V and E427K (itraconazole resistance) (Howard et al., 2009, Meneau et al., 2016); M172V, N248T, D255E (itraconazole and posaconazole resistance) (Prigitano et al., 2014); TR34/ L98H, M172V, N248T, D255E, E427K (itraconazole, posaconazole and isavuconazole resistance) (Abdolrasouli et al., 2015); and TR34/L98H, M172V, N248T, D255E, S297T, E427K, F495I (Abdolrasouli et al., 2015). It is also reported with isolates susceptible to azole antifungals (Snelders et al., 2010, Escribano et al., 2011, Alanio A et al., 2012, Buied et al., 2013, Abdolrasouli et al., 2015, Lavergne et al., 2015). Interestingly, some combinations of mutations associated with resistance have also been associated with susceptibility, including F46Y, M172V and E427K (Howard et al., 2009, Snelders et al., 2010, Escribano et al., 2011, Alanio A et al., 2012, Buied et al., 2013, Meneau et al., 2016). Following this publication, the F46Y and E427K mutations were also reported in a study researching *in vitro* resistance in human derived isolates of *A. fumigatus* in Australia using the EUCAST antifungal susceptibility microbroth dilution testing method

(Kidd et al., 2015). The isolate demonstrated intermediate susceptibility to itraconazole (2 µg/mL) and susceptibility to posaconazole (0.03 µg/mL) and voriconazole (0.25 µg/mL), with the *cyp51A* mutations F46Y, M172V and E427K. Based on *cyp51A* protein homology modelling using the crystal structure of the fungus *Saccharomyces cerevisiae*, the F46Y mutation has since been demonstrated to be at the *cyp51A* protein periphery and therefore is not considered important regarding the docking of azole molecules or the protein's structural integrity (Liu et al., 2016). It is most likely that non-*cyp51A* associated mechanisms contributed to the azole resistance in this isolate. The mutation G89E has not been reported for *A. fumigatus.*

This publication showed that azole resistance is not emerging amongst veterinary clinical isolates from Australia, the USA and Belgium. However, as this research detected azole resistance in an Australian canine isolate from the early 1990s and Kidd et al. (2015) detected ARAf in Australian human isolates, further investigation into ARAf in Australia was prompted, presented in Chapter 6 in an Australian focused study investigating azole resistance amongst environmental and clinical (human and additional veterinary) isolates of *A. fumigatus*.

5.5 References

ABDOLRASOULI, A., RHODES, J., BEALE, M. A., HAGEN, F., ROGERS, T. R., CHOWDHARY, A., MEIS, J. F., ARMSTRONG-JAMES, D. & FISHER, M. C. 2015. Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. *mBio,* 6**,** e00536.

ALANIO A, CABARET O, SITTERLÉ E, COSTA JM, BRISSE S, CORDONNIER C & BRETAGNEA S 2012. Azole preexposure affects the *Aspergillus fumigatus* population in patients. *Antimicrob Agents Chemother,* 56**,** 4948-4950.

BARRS, V. R., HALLIDAY, C., MARTIN, P., WILSON, B., KROCKENBERGER, M., GUNEW, M., BENNETT, S., KOEHLMEYER, E., THOMPSON, A., FLIEGNER, R., HOCKING, A., SLEIMAN, S., O'BRIEN, C. & BEATTY, J. A. 2012. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *The Veterinary Journal,* 191**,** 58-64.

BILLEN, F., GUIEU, L.-V., BERNAERTS, F., MERCIER, E., LAVOUÉ, R., TUAL, C., PEETERS, D. & CLERCX, C. 2010. Efficacy of intrasinusal administration of bifonazole cream alone or in combination with enilconazole irrigation in canine sino-nasal aspergillosis: 17 cases. *The Canadian Veterinary Journal,* 51**,** 164-168.

BUIED, A., MOORE, C. B., DENNING, D. W. & BOWYER, P. 2013. High-level expression of cyp51B in azole-resistant clinical *Aspergillus fumigatus* isolates. *Journal of Antimicrobial Chemotherapy,* 68**,** 512-4.

CHEN, J., LI, H., LI, R., BU, D. & WAN, Z. 2005. Mutations in the cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *Journal of Antimicrobial Chemotherapy,* 55**,** 31-37.

CLAEYS, S., LEFEBVRE, J. B., SCHULLER, S., HAMAIDE, A. & CLERCX, C. 2006. Surgical treatment of canine nasal aspergillosis by rhinotomy combined with enilconazole infusion and oral itraconazole. *The Journal of Small Animal Practice,* 47**,** 320-4.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) 2018. M59 Epidemiology cutoff values of antifungal susceptibility testing Clinical and Laboratory Standards Institute: 2nd ed. CLSI supplement M59, Wayne, PA.

CLINICAL LABOARTORY STANDARDS INSTITUTE (CLSI) 2017. M38, 3rd edition *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi.* Clinical and Laboratory Standards Institute: CLSI, Wayne, PA.

ESCRIBANO, P., RECIO, S., PELAEZ, T., BOUZA, E. & GUINEA, J. 2011. *Aspergillus fumigatus* strains with mutations in the *cyp51A* gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. *Antimicrobial Agents and Chemotherapy,* 55**,** 2460-2.

ESPINEL-INGROFF, A., CHOWDHARY, A., GONZALEZ, G. M., LASS-FLORL, C., MARTIN-MAZUELOS, E., MEIS, J., PELAEZ, T., PFALLER, M. A. & TURNIDGE, J. 2013. Multicenter study of isavuconazole MIC distributions and epidemiological cutoff values

for *Aspergillus* spp. for the CLSI M38-A2 broth microdilution method. *Antimicrobial Agents and Chemotherapy,* 57**,** 3823-8.

ESPINELL-INGROFF, A., DIEKEMA, D. J., FOTHERGILL, A., JOHNSON, E., PELAEZ, T., PFALLER, M. A., RINALDI, M. G., CANTON, E. & TURNIDGE, J. 2010. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Journal of Clinical Microbiology,* 48**,** 3251-3257.

EUCAST 2017. Antifungal agents breakpoint tables for interpretation of MICs. *Aspergillus spp.* Sweden: EUCAST.

FURROW, E. & GROMAN, R. P. 2009. Intranasal infusion of clotrimazole for the treatment of nasal aspergillosis in two cats. *Journal of the American Veterinary Medical Association,* 235**,** 1188-1193.

GOODALL, S. A., LANE, J. G. & WARNOCK, D. W. 1984. The diagnosis and treatment of a case of nasal aspergillosis in a cat. *The Journal of Small Anim Practice,* 25**,** 627-633.

HOWARD, S. J., CERAR, D., ANDERSON, M. J., ALBARRAG, A., FISHER, M. C., PASQUALOTTO, A. C., LAVERDIERE, M., ARENDRUP, M. C., PERLIN, D. S. & DENNING, D. W. 2009. Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerging Infectious Diseases,* 15**,** 1068-76.

KIDD, S. E., GOEMAN, E., MEIS, J. F., SLAVIN, M. A. & VERWEIJ, P. E. 2015. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses,* 58**,** 350-355.

LANTHIER, T. & CHALIFOUX, A. 1991. Enilconazole as an adjunct to the treatment of four cases of canine nasal aspergillosis. *The Canadian Veterinary Journal,* 32**,** 110-2.

LAVERGNE, R. A., MORIO, F., FAVENNEC, L., DOMINIQUE, S., MEIS, J. F., GARGALA, G., VERWEIJ, P. E. & LE PAPE, P. 2015. First description of azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation in France. *Antimicrobial Agents and Chemotherapy,* 59**,** 4331-5.

LIU, M., ZHENG, N., LI, D., ZHENG, H., ZHANG, L., GE, H. & LIU, W. 2016. cyp51A-based mechanism of azole resistance in Aspergillus fumigatus: Illustration by a new 3D Structural Model of Aspergillus fumigatus CYP51A protein. *Med Mycol,* 54**,** 400-8.

MENEAU, I., COSTE, A. T. & SANGLARD, D. 2016. Identification of *Aspergillus fumigatus* multidrug transporter genes and their potential involvement in antifungal resistance. *Med Mycol,* 54**,** 616-27.

PFALLER, M. A., DIEKEMA, D. J., GHANNOUM, M. A., REX, J. H., ALEXANDER, B. D., ANDES, D., BROWN, S. D., CHATURVEDI, V., ESPINEL-INGROFF, A., FOWLER, C. L., JOHNSON, E. M., KNAPP, C. C., MOTYL, M. R., OSTROSKY-ZEICHNER, L., SHEEHAN, D. J., WALSH, T. J. & SUBCOMMITTEE., C. L. S. I. A. T. 2009. Wild-type MIC distribution and epidemiological cutoff values for *Aspergillus fumigatus* and three triazoles as determined by the Clinical and Laboratory Standards Institute broth microdilution methods. *Journal of Clinical Microbiology,* 47**,** 3142-6.

PRIGITANO, A., VENIER, V., COGLIATI, M., DE LORENZIS, G., ESPOSTO, M. C. & TORTORANO, A. M. 2014. Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012. *Euro Surveillance,* 19**,** pii=20747.

REX, J. H., ALEXANDER, B. D., ANDES, D., ARTHINGTON-SKAGGS, B., BROWN, S. D., CHATURVELI, V., EPINELL-INGROFF, A., GHANNOUM, M. A., KNAPP, C. C., MOTYL, M. R., OSTROSKY-ZEICHNER, L., PFALLER, M., SHEEHAN, D. J. & WALSH, T. J. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi Approved Standard M38-A2. 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute.

RODRIGUEZ-TUDELA, J. L., ALCAZAR-FUOLI, L., MELLADO, E., ALASTRUEY-IZQUIERDO, A., MONZON, A. & CUENCA-ESTRELLA, M. 2008. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy,* 52**,** 2468-72.

SHARMAN, M., PAUL, A., DAVIES, D., MACKAY, B., SWINNEY, G., BARRS, V., ARTEAGA, A., ROBERTSON, I. D. & MANSFIELD, C. 2010. Multi-centre assessment of

mycotic rhinosinusitis in dogs: a retrospective study of initial treatment success (1998 to 2008). *Journal of Small Animal Practice,* 51**,** 423-427.

SHARP, N. J., SULLIVAN, M., HARVEY, C. E. & WEBB, T. 1993. Treatment of canine nasal aspergillosis with enilconazole. *Journal of Veterinary Internal Medicine,* 7**,** 40-3.

SNELDERS, E., KARAWAJCZYK, A., SCHAFTENAAR, G., VERWEIJ, P. E. & MELCHERS, W. J. 2010. Azole resistance profile of amino acid changes in *Aspergillus fumigatus CYP51A* based on protein homology modeling. *Antimicrobial Agents and Chemotherapy,* 54**,** 2425-2430.

STENGEL, C. Meticulous debridement as sole management for successful outcome in 6 dogs with sinonasal aspergillosis (SNA). 27th ECVIM-CA Congress, 14-16 September 2017 St. Julian's, Malta.

TALBOT, J. J., JOHNSON, L. R., MARTIN, P., BEATTY, J. A., SUTTON, D. A., BILLEN, F., HALLIDAY, C. L., GIBSON, J. S., KIDD, S., STEINER, J. M., UJVARI, B. & BARRS, V. R. 2014. What causes canine sino-nasal aspergillosis? A molecular approach to species identification. *The Veterinary Journal,* 200**,** 17-21.

TAMBORINI, A., ROBERTSON, E., TALBOT, J. J. & BARRS, V. R. 2016. Sinonasal aspergillosis in a British Shorthair cat in the UK. *Journal of Feline Medicine and Surgery Open Reports,* 2**,** 2055116916653775.

TOMSA, K., GLAUS, T. M., ZIMMER, C. & GREENE, C. E. 2003. Fungal rhinitis and sinusitis in three cats. *Journal of the American Veterinary Medical Association,* 222**,** 1380-4, 1365.

VERWEIJ, P. E., HOWARD, S. J., MELCHERS, W. J. & DENNING, D. W. 2009. Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. *Drug Resistance Updates,* 12**,** 141-7.

WIEDERHOLD, N. P., GIL, V. G., GUTIERREZ, F., LINDNER, J. R., ALBATAINEH, M. T., MCCARTHY, D. I., SANDERS, C., FAN, H., FOTHERGILL, A. W. & SUTTON, D. A.

2016. First detection of TR34 L98H and TR46 Y121F T289A *Cyp51* mutations in *Aspergillus fumigatus* isolates in the United States. *Journal of Clinical Microbiology,* 54**,** 168-71.

ZONDERLAND, J. L., STÖRK, C. K., SAUNDERS, J. H., HAMAIDE, A. J., BALLIGAND, M. H. & CLERCX, C. M. 2002. Intranasal infusion of enilconazole for treatment of sinonasal aspergillosis in dogs. *Journal of the American Veterinary Medical Association,* 221**,** 1421-1425.

Chapter 6. Investigation for *cyp51A* resistance in Australian environmental and clinical isolates of *Aspergillus fumigatus* sensu stricto

6.1 Introduction

Azole-resistant *Aspergillus fumigatus* (ARAf) have been identified worldwide as a major threat to the treatment of aspergillosis in people. Studies have demonstrated the relationship between the use of triazole fungicides in agricultural practice and the development of resistance in clinical patients treated with triazole therapeutics. Additionally, resistance can develop following prolonged use of azole therapeutics. The most common cause of ARAf are tandem repeats and/or single point mutations in the *cyp51A* gene, which is the target gene of triazole drugs. Although ARAf is recognised globally in human derived and environmental isolates, few studies investigated human clinical isolates from Australian, with one study identifying occasional ARAf in isolates from human patients, and reporting the TR_{34}/L98H mechanism or c*yp51A* point mutations in these isolates (Kidd et al., 2015). Chapter 5 presented the first study investigating ARAf in veterinary clinical isolates (dogs and cats) of Australian origin, finding *in vitro* azole resistance in a dog isolate from 1992. There are no reported Australian environmental investigations for ARAf. This chapter investigates the prevalence of ARAf amongst many clinical (human and veterinary) and environmental isolates in Australia, and utilises *cyp51A* sequencing and protein homology modelling to investigate resistant mechanisms.

6.2 Premise

This study from 2016 to 2017 was a collaboration with the Centre for Infectious Diseases and Microbiology Laboratory Services, ICPMR, New South Wales Health Pathology at Westmead Hospital. Given the limited number of Australian *A. fumigatus* isolates screened for ARAf (Kidd et al., 2015), and following my work in Chapter 5, I was keen to screen my collection of Australian isolates for ARAf.

This chapter presents findings on azole resistance in Australian environmental (azole-exposed and azole-naïve) and clinical (human and veterinary) isolates. A random selection of *A.*

fumigatus environmental isolates from Chapter 3, avian clinical isolates from Chapter 4 and clinical veterinary samples of *A. fumigatus* from dogs and cats are included. I collected 69 soil samples from public hospital grounds and New South Wales agricultural lands (viticulture and fruit crops). I received 25 soil samples from viticulture sites in Western Australia and four from Tasmania from Dr Fran Lopez and Dr Lincoln Harper (School of Science, Faculty of Science and Engineering, Curtin University), and three soil samples from agricultural crops in New South Wales from Dr Will Cuddy (NSW Department of Primary Industries) and Professor Robert Park (Faculty of Agriculture & Environment, University of Sydney).

A. fumigatus was isolated from soil using two methods. The first used a simple dilution technique (Snelders et al., 2009) and the second the heat activation method described in Chapter 3 (Nováková et al., 2014, Houbraken and Samson, 2006) to activate any *A. fumigatus* ascospores in the soil. *A. fumigatus* strains were isolated from azole-naive and azole-exposed soil by Prof Vanessa Barrs and myself. I analysed the macroscopic and microscopic morphologies of resulting cultures to confirm isolates were *Aspergillus* species (Samson et al., 2010) which were then sub-cultured for molecular identification using the partial beta-tubulin gene, to confirm their identity to species level as *A. fumigatus* (Glass and Donaldson, 1995). I identified all environmental and veterinary clinical isolates by amplifying the partial beta-tubulin gene.

The azole susceptibility screening test VIPcheck™ (AMT Plates, Balis Laboratorium V.O.F., Boven Leeuwen, the Netherlands) was used to examine all isolates for azole resistance. This test has high sensitivity (99%) and specificity (99%) for detecting ARAf amongst isolates of *A. fumigatus* (van der Linden et al., 2013, Arendrup et al., 2017). Environmental and veterinary *A. fumigatus* isolates were screened for azole resistance using the VIPcheck™ test by myself and Prof Vanessa Barrs. Any isolates demonstrating growth in drug wells on VIPcheck™ testing were further assessed by the colorimetric microbroth dilution antifungal susceptibility test Sensititre™ YeastOne™ YO10 assay (TREK Diagnostic Systems Ltd., OH, USA) to confirm or disprove resistant phenotype, based on recommended epidemiological cut-off values (ECVs) for *A. fumigatus* (Espinell-Ingroff et al., 2010, Clinical and Laboratory Standards Institute (CLSI), 2018).

Cyp51A sequencing was repeated for the presence of mutations in the gene for any isolates with ARAf or high MICs for voriconazole itraconazole or posaconazole (Chen et al., 2005). I performed *cyp51A* sequencing on an environmental isolate with high MIC against

voriconazole.

All human isolate DNA sequencing (partial beta-tubulin and *cyp51A*), VIPcheck™ testing, and all Sensititre™ YeastOne™ YO10 microbroth dilution antifungal testing included in this study were processed by our Westmead Hospital collaborators, Dr Shradha Subedi, Dr Catriona Halliday and Associate Professor Sharon Chen.

This research identified the resistant *cyp51A* genotype amongst isolates of *A. fumigatus* with azole resistant phenotypes. To demonstrate the *cyp51A* mutations could produce the resistant phenotype, a crystal structure homology model was designed by Prof David Hibbs and Ms Felcia Lai (Faculty of Pharmacy, the University of Sydney) based on published homology models for *A. fumigatus cyp51A* with the closest percentage match (*Saccharomyces cerevisiae cyp51A* and the *A. fumigatus cyp51B* model) (Liu et al., 2016, Hargrove et al., 2015).

6.3 Publication

The following publication has been re-formatted here to suit the style of this thesis. The publication's in-text references remain in the style required by the journal (superscript number form) and the publication associated tables, figures, references and supplementary data follow the main text. This research is presented in its original publication form in **Appendix 5**. A list of all other references referred to in this chapter outside of section **6.3 Publication** can be found at the chapter's end (see **6.5 References**).

Talbot J.J., Subedi S., Halliday C., Hibbs D., Lai F., Lopez-Ruiz F.J., Harper L., Park R.F., Cuddy W.S., Biswas C., Cooley L., Carter D., Sorrell T.C., Barrs V.R., Chen S.C-A. Surveillance for azole resistance in clinical and environmental isolates of Aspergillus fumigatus in Australia and cyp51A homology modelling of azole-resistant isolates. *Journal of Antimicrobial Chemotherapy*. 2018, dky187, https://doi.org/10.1093/jac/dky187.

Abstract

Background: The prevalence of azole resistance in *Aspergillus fumigatus* is uncertain in Australia. Azole exposure may select for resistance. We investigated the frequency of azole resistance in a large number of clinical and environmental isolates.

Methods: *A. fumigatus* isolates [148 human, 21 animal and 185 environmental strains from air (n = 6) and azole-exposed (n = 64) or azole-naive (n = 115) environments] were screened for azole resistance using the VIPcheck™ system. MICs were determined using the Sensititre™ YeastOne YO10 assay. Sequencing of The *Aspergillus cyp51A* gene and promoter region was performed for azole-resistant isolates, and *cyp51A* homology protein modelling undertaken.

Results: Non-WT MICs/MICs at the epidemiological cut-off value of one or more azoles were observed for 3/148 (2%) human isolates but not amongst animal, or environmental, isolates. All three isolates grew on at least one azole-supplemented well based on VIPcheck™ screening. For isolates 9 and 32, the itraconazole and posaconazole MICs were 1 mg/L (voriconazole MICs 0.12 mg/L); isolate 129 had itraconazole, posaconazole and voriconazole MICs of >16, 1 and 8 mg/L, respectively. Soil isolates from azole-exposed and azole-naive environments had similar geometric mean MICs of itraconazole, posaconazole and voriconazole ($P > 0.05$). A G54R mutation was identified in the isolates exhibiting itraconazole and posaconazole resistance, and the TR34/L98H mutation in the pan-azole-resistant isolate. *cyp51A* modelling predicted that the G54R mutation would prevent binding of itraconazole and posaconazole to the haem complex.

Conclusions: Azole resistance is uncommon in Australian clinical and environmental *A. fumigatus* isolates; further surveillance is indicated.

Introduction

Aspergillus fumigatus causes aspergillosis in humans and animals ranging from chronic forms to acute invasive aspergillosis (IA). IA, in particular, presents significant problems for haematological patients, with mortalities of >50%.[1,2] Antifungal triazoles are first-line agents for managing these infections and preventing fungal diseases in agriculture and construction.[3] The emergence of azole-resistant strains of *A. fumigatus* (ARAf) is thus concerning, being associated with treatment failure and resistance, and may be particularly relevant in haematology patients, many of whom have prior exposure to azoles.[2,4–6] Selection pressure for ARAf has also been linked to exposure to agricultural azole-based fungicides, the demethylation inhibitors (DMIs).[5–7] Major mechanisms of azole resistance in ARAf include SNPs in the *A. fumigatus cyp51A* gene, alone or combined with tandem repeats (TRs) in the promoter region.[8–10]

The prevalence of ARAf varies with geographical region and is highest in Europe (3%–26%).[5,8,10] In Australia, while ARAf isolates have been identified,[11] their prevalence is uncertain. To better understand the burden of ARAf, we investigated the frequency of resistance to itraconazole, voriconazole and posaconazole among clinical and environmental *A. fumigatus* isolates.

Methods

Human isolates of *A. fumigatus* ($n = 148$) were obtained from culture collections at Westmead and Royal Hobart Hospitals from 2015 to 2017 (Table S1, available as Supplementary data at *JAC* Online). Of these, 35 isolates were from patients with physician-ascribed IA using established criteria where appropriate (see Table S1).[12] Twenty-one animal isolates (School of Veterinary Science, University of Sydney, 2013–17; Table S1) and 185 environmental isolates from azole-naive ($n = 115$) or azole-exposed ($n = 64$) habitats and ambient air ($n = 6$) (2013–17; Table S2) were also studied.

For isolation of *A. fumigatus*, soil samples were stored at 4°C and processed within 1 week (method 1),[13] with 100 μL of soil suspension incubated at 45°C on malt extract agar containing chloramphenicol (MEASC). Early experiments showed that for azole-treated soils, *A. fumigatus* yields were low and samples were reprocessed using 'method 2' as previously described.[14] Briefly, two sets of 5× serial 1:10 dilutions of soil were analysed. One set was heat treated at 75°C for 30 min and 100 μL from each dilution was incubated at 37°C on MEASC for 21 days. Colonies were identified as *A. fumigatus* species complex by standard morphological methods.[15] Identity was confirmed as *A. fumigatus sensu stricto* by *β-tubulin* gene sequencing.[16]

To screen for azole resistance, *Aspergillus* isolates were inoculated onto 4-well VIPcheck™ plates (Balis Laboratorium VOF, Boven Leeuwen, The Netherlands) with wells supplemented with itraconazole (4 mg/L), voriconazole (2 mg/L) or posaconazole (0.5 mg/L), or unsupplemented.[17] Isolates growing only in the control well were considered azole susceptible. Isolates that grew on any azole-containing well underwent re-examination and susceptibility testing using the Sensititre™ YeastOne YO10 system (TREK Diagnostics Systems Ltd, OH, USA).[18] Both Sensititre™ and VIPcheck™ testing was repeated for isolates that grew on azole-supplemented wells.

Susceptibility testing was performed on all human isolates and randomly selected animal ($n = 11$, 52%) and environmental ($n = 41$, 22%) isolates according to the manufacturer's instructions and following CLSI methodology (Third Edition: M38).[18] MICs or minimum effective concentrations (MECs) were read after 48 h of incubation and determined by visual inspection. *Candida parapsilosis* ATCC 22019 (GenBank reference NRRL Y-12969) was the quality control, and *A. fumigatus* ATCC 204305 (GenBank reference KC689329, KU729126 and KU897017) was the reference strain.[18] MICs or MECs were assessed using established criteria, with MICs read at 100% inhibition, i.e. no signs of visual growth.[18] Interpretative criteria based on epidemiological cut-off values (ECVs) were: for itraconazole and voriconazole, an MIC of ≤1 mg/L was WT and ≥2 mg/L was non-WT (NWT); and for posaconazole, an MIC of ≤0.25 mg/L was WT and >0.5 mg/L was NWT.[19,20] Geometric mean (GM) MICs/MECs were calculated for WT isolates. Standard deviations and *t*-scores compared GM MICs for groups of isolates with significance set at $P \leq 0.05$. For isolates with NWT MICs, the *Aspergillus cyp51A* gene and promoter region were amplified and sequenced.[21] Sequences were aligned with GenBank reference sequence AF222068.[21]

The *A. fumigatus cyp51A* sequence AF338659[22] was obtained from the universal protein resource (http://www.uniprot.org). Homology modelling was *inter alia cyp51B*-based with the crystal structure of *A. fumigatus cyp51B* retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org) as it returned the highest sequence identity and BLAST score (identification no. 5FRB: resolution 2.99 Å, max score 660, total score 660, query cover 92%, identity 65%). Of five homology models of the *cyp51A* gene built using Schrödinger's Prime,[23] the best model (data not shown) was prepared for molecular docking (see below).

The single-residue mutation G54R was tested with 2D structures of itraconazole, posaconazole and voriconazole and drawn (Schrödinger Release 2017-3; LigPrep LLC, New York). Docking of the three triazoles was carried out via Glide with extra precision (XP),[24] and the potential energy in different conformations with an open and closed binding cavity was calculated via MacroModel (Schrödinger Release 2017-3).

Results

Environmental sampling

In addition to culture collection isolates included in this study [ambient air ($n = 6$) and soil ($n = 20$)] a further 159 *A. fumigatus* environmental cultures were isolated from soil. Method 1 yielded 91 *A. fumigatus* isolates from azole-naive soils ($n = 90$) and azole-exposed soils ($n = 1$). Method 2 yielded an additional 68 isolates from azole-naive soils ($n = 5$) and azole-exposed soils ($n = 63$).

Resistance screening

Five isolates (one avian and four from azole-exposed soil) grew on the itraconazole-containing well of the VIPcheck™ plate. Of 148 human isolates, isolate 129 grew on all azole-supplemented wells, and isolates 9 and 32 grew on posaconazole- and itraconazole-containing wells. Isolates 32 and 129 were from patients who had received prior fluconazole as antifungal prophylaxis during the preceding 6 month period, whilst isolate 9 was from an azole-naive patient. All three patients had invasive pulmonary aspergillosis.[12]

Susceptibility testing

Table 1 summarizes the MIC/MEC values for the study isolates (see Tables S1 and S2 for individual isolate results). Of 148 human isolates, 145 had WT MICs below the ECV for the drugs tested with GM MICs of itraconazole, posaconazole, voriconazole and amphotericin B of 0.12, 0.06, 0.23 and 1.04 mg/L, respectively (MIC_{90} values were 0.25, 0.12, 0.5 and 2.0 mg/L, respectively). Of three isolates that had MICs at the ECV or NWT MICs (Table S1), isolates 9 and 32 had an MIC of 1 mg/L of posaconazole and itraconazole (voriconazole MIC 0.12 mg/L). Isolate 129 had MICs of >16, 8 and 1 mg/L of itraconazole, voriconazole and posaconazole, respectively. However, the avian isolate that grew on the itraconazole-containing well of the VIPcheck™ plate had a WT itraconazole MIC (0.06 mg/L). The assignment of MIC results as WT or NWT/MIC at the ECV of clinical isolates was concordant with results obtained by VIPcheck™ screening for 99% of clinical isolates.

All 41 environmental isolates had WT MIC values of the azoles (Table 1); GM MICs of itraconazole, posaconazole and voriconazole were 0.13, 0.05 and 0.29 mg/L, respectively (MIC_{90} 0.25, 0.12 and 0.5 mg/L). All four isolates that grew on the itraconazole-containing agar well of the VIPcheck™ plate had WT MICs of itraconazole [0.06 ($n = 3$) and 0.12 ($n = 1$)

mg/L]. Comparison of GM MICs, MIC_{50} values and MIC_{90} values between 'azole-exposed' and 'azole-naive' isolates showed these were not statistically different (data not shown: posaconazole, $P > 0.05–0.1$; and itraconazole and voriconazole, $P > 0.2$).

Cyp51A sequencing and modelling

Cyp51A mutations were identified in the three isolates with NWT MICs or MICs at the ECV (see Table S1) (GenBank accession numbers MG687254–MG687257). Isolates 9 and 32 had SNPs present at nucleotide position 160 (GGG to AGG and CGG, respectively), resulting in a G54R substitution. Isolate 32 had another SNP at nucleotide position 744 (AAT to AAA), resulting in N248K. Isolate 129 carried a TR_{34}/L98H mutation at nucleotide position 323. Modelling G54R indicated that replacing the short side-chain glycine (G) with arginine (R) allowed the formation of hydrogen bonds with T50 and S493, which closes off binding channel access (Figure 1). This closed conformation stabilized the model by 84.47 kcal/mol with high likelihood that the large triazole structures (itraconazole and posaconazole but not voriconazole) would be prevented from entering the channel.

Discussion

This large study found isolates with NWT MICs to be uncommon in Australian human clinical isolates (2.0%), and at the lower end of that seen globally (0%–26%), contrasting particularly with that in the Netherlands.[5,8,10] Of note, ARAf was absent amongst animal isolates. These data suggest that treatment guidelines currently recommending azoles as preferred therapy for IA remain appropriate in Australia.[3]

Also notable was the apparent absence of ARAf in the environment, including azole-exposed habitats. Environmental studies have reported a broad range of prevalence of ARAf (0%–91%), with the highest prevalence in the Netherlands, where most resistant isolates carry the TR_{34}/L98H mechanism.[7] The implications of our results for agricultural practice, although uncertain, raise questions regarding the impact of different agricultural practices between Australia and Europe including percentage of crop area treated, number and amount of applications/crop, and type and rotation of DMIs used. Composts, which have yielded a high percentage of environmental ARAf in Europe,[25] are not used in broad-acre farming in Australia. Additionally, compared with Western Europe, the Australian climate has prolonged periods of dryness or drought, where the need for DMIs is greatly reduced. In Australia, the most

commonly used azole fungicides for grape crops include difenoconazole, myclobutanil, penconazole, tebuconazole and tetraconazole and for grain crops cyproconazole, epoxiconazole, propiconazole, prothioconazole and tebuconazole.[26] Azole fungicide and medical practices of different locations worldwide should be compared to further our understanding of the variation in ARAf prevalence rates between regions.

The agar-based VIPcheck™ screening test is well established to have sufficiently high sensitivity and specificity for predicting azole resistance.[27] In the present study, the test proved useful in screening for resistance with no false 'susceptible' results; all isolates with NWT MICs or with MICs at the ECV were observed to grow on the plates. The VIPcheck™ plates overcalled 'resistance' for five isolates (5/8 isolates, specificity 97.5%). This is reasonable for a screening test which must have a high negative predictive value. As the VIPcheck™ test does not measure MICs, all isolates that grow on azole-supplemented agar should be tested for antifungal susceptibility.

The G54R mutation, identified here in two isolates, is known to confer differential azole resistance in *A. fumigatus.*[9] One of our isolates with the G54R mutation had a second mutation leading to amino acid substitution N248K; however, this also occurs in azole-susceptible *A. fumigatus* isolates with no documented link to resistance.[9] Pan-resistance in isolate 129 was through the TR_{34}/L98H mutation, consistent with existing literature.[10] Of note, both TR_{34}/L98H and G54R mutations have been linked to environmental acquisition,[7,10] and were identified previously in three other Australian clinical isolates.[11]

Our homology modelling results using a *cyp51B A. fumigatus* model are consistent with previous observations in non-*Aspergillus cyp51A* models that the G54R mutation can prevent docking of the long-tailed azoles itraconazole and posaconazole but not of the more compact voriconazole molecule.[28]

Recovery of *A. fumigatus* from soil found that method 2, which uses heat-shock to inactivate asexual spores, gave a higher yield of isolates from azole-treated soils. Although no ARAf was found, the survival of sexual spores in azole-treated soil could serve as a potential reservoir for resistance development; in the laboratory setting, sexual reproduction has been implicated in the development of *cyp51A*-mediated azole resistance.[25]

A limitation of the present study was that clinical data were not collected. This prevented more

precise determination of ARAf incidence amongst different groups of azole-exposed or azole-naive patients. About half of the cystic fibrosis patients in the Centre for Infectious Diseases and Microbiology Laboratory Services receive intermittent azole therapy (usually itraconazole) (S. C.-A. Chen, unpublished data). Other isolates were recovered from disparate groups of patients with unknown azole exposure. Whilst it is possible that the low prevalence observed is related to potential inhomogeneity in the patient population, the fact that we only observed three 'azole-resistant' isolates would limit any statistically meaningful data. We acknowledge the importance of prospective studies to investigate azole resistance amongst specific patient groups.

In conclusion, ARAf appears to be uncommon in Australia, which indicates that the preferred therapy for aspergillosis does not need to be changed. However, continued surveillance is warranted to pre-empt the emergence of ARAf strains and to inform management strategies.

Acknowledgements

We wish to thank Professor Paul Verweij and the Department of Medical Microbiology at the Institute for Molecular Life Sciences at Radboud UMC, Nijmegen, The Netherlands, and Professor Oliver Bader at the Institute of Medical Microbiology, University Medical Center Goettingen, Göttingen, Germany, for providing assistance with aligning nucleotide positions to pinpoint mutation locations.

Publication Figures

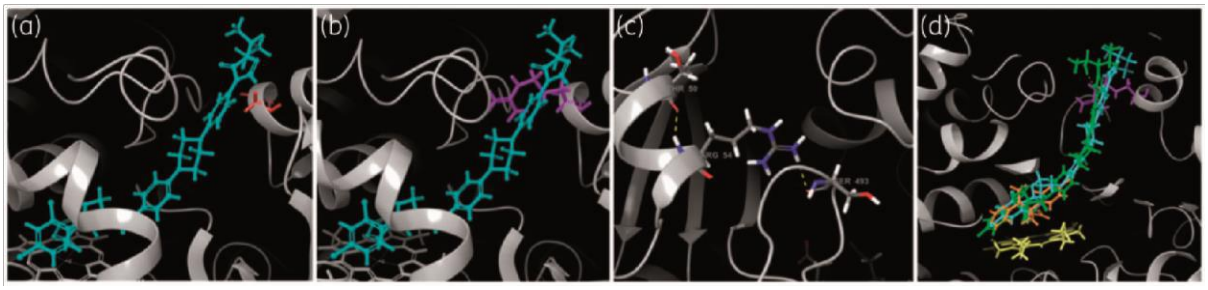


Figure 1. *A. fumigatus cyp51B* model demonstrating binding to the active site of *cyp51A* and the H-bond interaction. Itraconazole (blue) binding in the active site of *cyp51A*: (a) amino acid G54 (red) and (b) mutated G54R (purple). (c) Hydrogen bonding (yellow dashed lines) between T50, G54R and S493 (H-bond length: T50 and G54R, 1.99 Å; and S493 and G54R, 2.28 Å).

(d) Voriconazole (orange), posaconazole (green) and itraconazole (blue) in the binding cavity; residue G54R (purple) and the haem group (yellow) are shown.

Table 1. Antifungal susceptibilities [MIC or MEC[a] values (mg/L)] of 147 human,[b] 11 animal and 41 environmental isolates of *A. fumigatus*, according to origin of isolates

	AMB	ANF	CAS	FLC	ITC	MIF	POS	VRC
GM MIC/MEC								
human, invasive (35)	1.2	0.02	0.04	184.35	0.22	0.01	0.11	0.28
human, colonization (112)	0.99	0.02	0.03	195.85	0.12	0.01	0.05	0.22
animal (11)	1.13	0.02	0.04	256	0.12	0.02	0.04	0.34
azole-exposed soil (23)[c]	1.2	0.02	0.04	246	0.13	0.01	0.06	0.27
azole-naive soil (12)	1.06	0.02	0.04	256	0.12	0.02	0.04	0.31
air (6)	2	0.015	0.04	228	0.15	0.01	0.05	0.25
MIC/MEC$_{90}$								
human, invasive (35)	2	0.03	0.12	>256	0.5	0.015	0.5	0.5
human, colonization (112)	2	0.03	0.06	>256	0.25	0.03	0.12	0.5
animal (11)	2	0.03	0.06	>256	0.25	0.03	0.12	0.5
azole exposed soil (23)[c]	2	0.03	0.06	256	0.25	0.015	0.12	0.5
azole-naive soil (12)	1	0.03	0.06	>256	0.25	0.03	0.12	0.5
air (6)	2	0.015	0.06	256	0.25	0.015	0.06	0.5
MIC/MEC range								
human, invasive (35)	0.25–8	<0.015–0.06	0.008–0.5	64 to >256	0.06 to .16	<0.008–0.03	0.015–1	0.06–8
human, colonization (112)	0.25–2	<0.015–0.12	<0.008–0.12	64 to >256	<0.015–0.5	<0.008–0.03	<0.008–0.25	0.03–1
animal (11)	0.5–2	0.015–0.03	0.015–0.06	256 to >256	0.03–0.5	0.008–0.03	0.015–0.25	0.25–0.5
azole-exposed soil (23)[c]	0.5–2	0.015–0.06	0.015–0.06	128 to >256	0.03–0.5	0.008–0.03	0.015–0.25	0.12–1.0
azole-naive soil (12)	1–2	0.015–0.06	0.03–0.06	256 to >256	0.03–0.25	0.008–0.03	0.015–0.12	0.25–0.5
air (6)	2	0.015	0.03–0.06	128–256	0.12–0.25	0.008–0.015	0.03–0.06	0.06–0.5

AMB, amphotericin B; ANF, anidulafungin; CAS, caspofungin; FLC, fluconazole; ITC, itraconazole; MIF, micafungin; POS, posaconazole; VRC, voriconazole. Numbers in brackets refer to the numbers of isolates tested. [a] MECs are reported for echinocandin drugs (anidulafungin, caspofungin and micafungin). [b] Disease state information was not available for one human isolate (isolate number 24). MICs for this isolate were (in mg/L): amphotericin B, 1; anidulafungin, 0.015; caspofungin, 0.06; fluconazole, 256; itraconazole, 0.25; micafungin, 0.008; posaconazole, 0.12; and voriconazole, 0.25. [c] DMIs used on azole-exposed soil isolates included cyproconazole (n=3), myclobutanil (n=4), tebuconazole and epoxiconazole (n=10), tebuconazole and myclobutanil (n=1), tebuconazole and penconazole (n=3), and tetraconazole and myclobutanil (n=2).

Publication References

1 Brown GD, Denning DW, Gow NA et al. Hidden killers: human fungal infections. Sci Transl Med 2012; 4: 165rv13.

2 Steinmann J, Hamprecht A, Vehreschild MJ et al. Emergence of azole resistant invasive aspergillosis in HSCT recipients in Germany. J Antimicrob Chemother 2015; 70: 1522–6.

3 Patterson TF, Thompson IIIGR, Denning DW et al. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis 2016; 63: e1–60.

4 Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. The global problem of antifungal resistance: prevalence, mechanisms, and management. Lancet Infect Dis 2017; 17: e383–92.

5 van der Linden JW, Arendrup MC, Warris A et al. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. Emerg Infect Dis 2015; 21: 1041–4.

6 van der Linden JW, Snelders E, Kampinga GA et al. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. Emerg Infect Dis 2011; 17: 1846–54.

7 Verweij PE, Snelders E, Kema GHJ et al. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? Lancet Infect Dis 2009; 9: 789–95.

8 Garcia-Rubio R, Cuenca-Estrella M, Mellado E. Triazole resistance in *Aspergillus* species: an emerging problem. Drugs 2017; 77: 599–613.

9 Dudakova A, Spiess B, Tangwattanachuleeporn M et al. Molecular tools for the detection and deduction of azole antifungal drug resistance phenotypes in *Aspergillus* species. Clin Microbiol Rev 2017; 30: 1065–91.

10 Chowdhary A, Sharma C, Meis JF. Azole-resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. J Infect Dis 2017; 216 Suppl 3: S436–44.

11 Kidd SE, Goeman E, Meis JF et al. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. Mycoses 2015; 58: 350–5.

12 De Pauw B, Walsh TJ, Donnelly JP et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 2008; 46: 1813–21.

13 Snelders E, Huis in't Veld RA, Rijs AJM et al. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazole. Appl Environ Microbiol 2009; 75: 4053–7.

14 Nováková´ A, Hubka V, Dudová´ Z et al. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. Fungal Divers 2014; 64: 253–74.

15 Samson RA, Houbraken J, Thrane U et al. Food and Indoor Fungi. Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures, 2010.

16 Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 1995; 61: 1323–30.

17 van der Linden JWM, Arendrup MC, van der Lee HAL et al. Azole containing agar plates as a screening tool for azole resistance of *Aspergillus fumigatus*. Mycoses 2009; 52: 19.

18 Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi—Third Edition: M38. CLSI, Wayne, PA, USA, 2017.

19 Espinell-Ingroff A, Diekema DJ, Fothergill A et al. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). J Clin Microbiol 2010; 48: 3251–7.

20 Clinical and Laboratory Standards Institute. Epidemiology Cutoff Values for Antifungal Susceptibility Testing—Second Edition: M59. CLSI, Wayne, PA, USA, 2018.

21 Chen J, Li H, Li R et al. Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. J Antimicrob Chemother 2005; 55: 31–7.

22 Mellado E, Diaz-Guerra TM, Cuenca-Estrella M et al. Identification of two different 14-a sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol 2001; 39: 2431–8.

23 Jacobson MP, Pincus DL, Rapp CS et al. A hierarchical approach to allatom protein loop prediction. Proteins 2004; 55: 351–67.

24 Friesner RA, Murphy RB, Repasky MP et al. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein ligand complexes. J Med Chem 2006; 49: 6177–96.

25 Zhang J, Snelders E, Zwaan BJ et al. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. MBio 2017; 8: e00791-17.

26 Australian Wine Research Institute. Agrochemicals Registered for Use in Australian Viticulture. 2017. https://www.awri.com.au/wp-content/uploads/ agrochemical_booklet.pdf.

27 Arendrup MC, Verweij PE, Mouton JW et al. Multicentre validation of 4-well azole agar plates as a screening method for detection of clinically relevant azole-resistant *Aspergillus fumigatus*. J Antimicrob Chemother 2017; 72: 3325–33.

28 Fraczek MG, Bromley M, Bowyer P. An improved model of the *Aspergillus fumigatus CYP51A* protein. Antimicrob Agents Chemother 2011; 55: 2483–6.

Publication Supplementary Data

Table S1. Isolate details for human (n=148) and animal (n=21) patients from whom *Aspergillus fumigatus* was cultured. MIC, or minimum effective concentration (for echinocandins), values are provided for 159 isolates.

Isolate Number	**Source isolate**	**Disease state (site of infection)***	**MICs or MECs^ (mg/L)**								
			AMB	**ANF**	**CAS**	**FLC**	**5FC**	**ITC**	**MIF**	**POS**	**VRC**
Human isolates											
1	Sputum	I (lung)	1	<0.015	0.015	256	0.5	0.12	<0.008	0.03	0.12
2	Pleural fluid	C	1	0.015	0.015	128	32	0.12	0.015	0.06	0.25
3	Lung tissue	I (lung)	0.5	0.015	0.015	128	2	0.06	<0.008	0.03	0.12
4	BAL fluid	I (lung)	1	0.015	0.015	128	1	0.06	0.008	0.03	0.12
5	BAL fluid	I (lung)	1	0.015	0.008	>256	16	0.12	0.008	0.03	0.25
6	BAL fluid	I (lung)	0.25	<0.015	0.015	256	1	0.12	<0.008	0.06	0.12
7	Parotid gland	I (soft tissue and parotid)	2	0.015	0.015	>256	2	0.12	0.008	0.06	0.25
8	Bronchial tissue	I (lung)	0.25	0.015	0.008	128	1	0.06	<0.008	0.03	0.06
9#	BAL fluid	I (lung)	1	0.03	0.06	64	32	1	0.015	1	0.12
10	Sputum	I (lung)	0.5	0.03	0.015	128	0.5	0.12	0.015	0.06	0.12
11	Bronchial tissue	I (lung)	0.5	0.015	0.03	256	2	0.12	0.008	0.06	0.25
12	Lung tissue	I (lung)	0.5	0.015	0.03	256	32	0.06	<0.008	0.015	0.12
13	Sinus tissue	I (sinus, bone)	2	0.03	0.03	>256	32	0.5	0.015	0.25	0.5
14	Lung tissue	I (lung)	1	0.015	0.06	>256	32	0.5	0.015	0.25	0.5
15	Sputum	I (lung)	2	0.03	0.03	>256	16	0.25	0.015	0.12	0.25

16	Mastoid tissue	I (bone, sinus, ear)	8	0.015	0.015	>256	16	0.12	0.015	0.06	0.25
17	Sputum	I (lung)	2	0.03	0.03	>256	16	0.25	0.008	0.12	0.25
18	BAL fluid	I (lung)	2	0.015	0.06	>256	16	0.25	0.008	0.12	0.25
19	BAL fluid	I (lung)	4	0.06	0.06	>256	64	0.5	0.015	0.25	0.5
20	Mastoid tissue	I (ear, bone)	2	0.03	0.06	256	8	0.12	0.015	0.03	0.25
21	Sputum	I (lung)	2	0.03	0.06	>256	4	0.5	<0.008	0.25	0.5
22	Pleural fluid	I (lung)	1	<0.015	0.015	256	16	0.5	0.008	0.5	1
23	Lung Tissue	I (lung)	2	0.015	0.06	256	8	0.25	0.008	0.12	0.5
24	NA	NA	1	0.015	0.06	256	32	0.25	0.008	0.12	0.25
25	Lung tissue	I (lung)	1	0.015	0.03	64	32	0.25	0.008	0.12	0.25
26	Sputum	C	1	0.03	0.06	256	16	0.12	0.015	0.03	0.25
27	Abdominal wall tissue	I (soft tissue, muscle)	1	0.015	0.12	256	32	0.12	0.015	0.03	0.25
28	Cornea	I (eye)	2	0.015	0.06	256	32	0.25	0.015	0.12	0.25
29	Sputum	I (lung)	1	0.015	0.06	>256	64	0.25	0.015	0.12	0.5
30	BAL fluid	I (lung)	4	0.03	0.25	>256	64	0.5	0.015	0.25	0.25
31	Sputum	I (lung)	1	0.06	0.5	>256	>64	0.5	0.03	0.25	0.5
32#	Sputum	I (lung)	1	0.03	0.03	256	32	1	0.015	1	0.12
33	Sputum	C	1	<0.015	0.015	256	4	0.03	<0.008	0.015	0.12
34	Sputum	C	0.5	<0.015	0.015	256	0.5	<0.015	<0.008	<0.008	0.06
35	Sputum	C	0.5	<0.015	0.015	256	1	0.03	<0.008	0.015	0.12
36	Sputum	C	0.5	<0.015	0.015	256	2	0.03	<0.008	0.015	0.12
37	Sputum	C	0.5	<0.015	0.015	128	128	0.015	<0.008	<0.008	0.06
38	Sputum	C	0.5	<0.015	0.008	256	0.5	0.03	<0.008	0.015	0.06
39	Sputum	C	0.5	<0.015	0.008	128	1	0.03	<0.008	0.015	0.06
40	Sputum	C	0.5	<0.015	0.008	128	0.5	0.06	<0.008	0.015	0.06
41	Sputum	C	0.25	<0.015	0.008	128	0.25	0.015	<0.008	0.008	0.03

42	Sputum	C	0.5	<0.015	0.008	256	1	0.06	<0.008	0.03	0.12
43	Sputum	C	0.25	<0.015	0.015	128	0.5	0.03	<0.008	0.015	0.06
44	Sputum	C	0.25	<0.015	0.015	128	0.5	0.03	<0.008	0.015	0.06
45	Sputum	C	0.5	0.015	0.015	256	1	0.12	<0.008	0.03	0.12
46	Sputum	C	0.25	0.015	0.015	64	1	0.03	<0.008	0.015	0.06
47	Sputum	C	1	0.015	0.06	256	16	0.06	0.015	0.03	0.12
48	Sputum	C	0.25	<0.015	<0.008	256	0.25	0.06	<0.008	0.03	0.06
49	Sputum	C	0.5	0.015	<0.008	256	0.5	0.12	<0.008	0.06	0.12
50	Sputum	C	0.5	0.015	0.03	256	0.5	0.06	<0.015	0.03	0.12
51	Sputum	C	0.5	<0.015	0.015	128	0.25	0.03	<0.008	0.015	0.06
52	Sputum	C	0.25	<0.015	0.015	256	0.25	0.06	<0.008	0.03	0.06
53	Sputum	C	0.25	<0.015	<0.008	128	1	0.06	<0.008	0.03	0.06
54	Sputum	C	0.25	<0.015	<0.008	128	0.25	0.06	<0.008	0.03	0.06
55	Sputum	C	0.5	<0.015	0.008	256	0.5	0.12	<0.008	0.015	0.25
56	Sputum	C	1	0.015	0.12	256	4	0.25	0.015	0.12	0.25
57	Sputum	C	0.25	<0.015	0.015	256	4	0.12	<0.008	0.06	0.12
58	Sputum	C	0.25	<0.015	<0.008	64	0.25	0.06	<0.008	0.03	0.06
59	Sputum	C	0.25	<0.015	<0.008	256	0.5	0.03	<0.008	0.015	0.06
60	Sputum	C	0.5	<0.015	0.015	256	4	0.03	<0.008	0.03	0.12
61	Sputum	C	1	0.015	0.008	256	32	0.03	<0.008	0.03	0.25
62	Sputum	C	0.5	0.015	0.015	256	4	0.25	0.015	0.12	0.25
63	Sputum	C	1	<0.015	0.015	256	32	0.25	<0.008	0.12	0.25
64	Sputum	C	1	0.03	0.015	256	64	0.12	<0.008	0.12	0.25
65	Sputum	C	2	0.015	0.03	256	1	0.25	<0.008	0.12	0.25
66	Sputum	C	1	0.015	0.015	256	32	0.25	<0.008	0.12	0.25
67	Sputum	C	1	<0.015	<0.0008	256	32	0.25	<0.008	0.12	0.25
68	Sputum	C	1	0.015	0.015	256	4	0.25	0.008	0.12	0.5
69	Sputum	C	1	0.015	0.015	256	4	0.25	0.008	0.12	0.25

70	Sputum	C	1	0.03	0.03	256	32	0.5	0.008	0.12	0.5
71	Sputum	C	1	0.015	0.03	128	16	0.25	0.008	0.25	0.25
72	Sputum	C	1	0.015	0.015	128	32	0.25	0.008	0.12	0.25
73	Sputum	C	2	0.015	0.03	256	16	0.25	0.008	0.12	0.5
74	Sputum	C	1	0.015	0.03	256	16	0.25	0.008	0.12	0.25
75	Sputum	C	1	0.015	0.03	128	16	0.25	0.008	0.12	0.25
76	Sputum	C	0.25	0.015	0.015	128	1	0.25	0.008	0.06	0.12
77	Sputum	C	1	0.015	0.03	128	32	0.25	0.008	0.06	0.25
78	Sputum	C	1	0.015	0.03	128	16	0.25	0.008	0.12	0.5
79	Sputum	C	0.25	0.015	0.015	128	1	0.12	0.008	0.06	0.12
80	Sputum	C	0.25	0.015	0.015	128	0.5	0.12	0.008	0.06	0.12
81	Sputum	C	1	0.12	0.06	256	2	0.25	0.015	0.06	0.25
82	Sputum	C	2	0.03	0.015	256	32	0.25	0.008	0.12	0.25
83	Sputum	C	0.25	0.06	0.03	256	1	0.12	0.008	0.06	0.12
84	Sputum	C	0.5	0.015	0.03	64	1	0.25	0.008	0.12	0.25
85	Sputum	C	2	0.03	0.06	256	16	0.25	0.008	0.12	0.25
86	Sputum	C	0.5	0.015	0.015	128	4	0.25	0.008	0.06	0.12
87	Sputum	C	1	0.03	0.03	128	2	0.25	0.008	0.12	0.25
88	Sputum	C	2	0.03	0.012	>256	32	0.12	0.03	0.06	0.25
89	Sputum	C	1	0.015	0.03	>256	8	0.12	0.03	0.03	1
90	Sputum	C	2	0.015	0.03	>256	64	0.12	0.03	0.06	0.5
91	Sputum	C	2	0.03	0.03	>256	64	0.12	0.03	0.06	0.5
92	Sputum	C	2	0.015	0.03	>256	64	0.12	0.03	0.03	0.5
93	Sputum	C	2	0.03	0.06	256	64	0.12	0.03	0.03	0.5
94	Sputum	C	2	0.015	0.03	>256	16	0.12	0.015	0.06	0.5
95	Sputum	C	2	0.015	0.06	>256	64	0.12	0.03	0.06	0.5
96	Sputum	C	2	0.015	0.015	>256	64	0.12	0.015	0.06	0.25
97	Sputum	C	2	0.03	0.06	256	32	0.06	0.03	0.015	0.25

98	Sputum	C	2	0.03	0.06	>256	32	0.12	0.03	0.03	0.5
99	Sputum	C	2	0.015	0.06	256	32	0.12	0.015	0.03	0.25
100	Sputum	C	2	0.015	0.06	256	32	0.12	0.03	0.03	0.5
101	Sputum	C	2	0.015	0.03	>256	16	0.12	0.015	0.06	0.5
102	Sputum	C	2	0.03	0.06	256	32	0.25	0.03	0.06	0.25
103	Sputum	C	2	0.015	0.03	>256	64	0.25	0.015	0.12	1
104	Sputum	C	2	0.03	0.06	256	64	0.16	0.03	0.06	0.25
105	Sputum	C	2	0.015	0.06	256	8	0.25	0.03	0.06	0.25
106	Sputum	C	2	0.015	0.015	256	16	0.12	0.008	0.03	0.5
107	Sputum	C	2	0.015	0.03	128	32	0.06	0.015	0.03	0.25
108	Sputum	C	2	0.015	0.015	256	4	0.12	0.008	0.03	0.25
109	Sputum	C	2	0.015	0.03	>256	32	0.12	0.015	0.03	0.25
110	Sputum	C	2	0.015	0.015	128	32	0.06	0.008	0.03	0.25
111	Sputum	C	2	0.015	0.03	256	64	0.12	0.008	0.03	0.25
112	Sputum	C	2	0.015	0.03	256	32	0.06	0.008	0.03	0.25
113	Sputum	C	2	0.015	0.015	128	16	0.03	0.015	0.03	0.25
114	Sputum	C	2	0.015	0.03	256	32	0.12	0.015	0.03	0.12
115	Sputum	C	2	0.015	0.03	>256	32	0.12	0.008	0.03	0.12
116	Sputum	C	2	0.015	0.015	256	32	0.06	<0.008	0.015	0.25
117	Sputum	C	2	0.015	0.015	256	64	0.03	0.008	0.015	0.25
118	Sputum	C	2	0.015	0.008	>256	32	0.06	0.008	0.03	0.25
119	Sputum	C	1	0.015	0.015	256	64	0.03	0.015	0.015	0.25
120	Sputum	C	2	0.03	0.008	>256	64	0.06	0.008	0.03	0.25
121	Sputum	C	2	0.015	0.03	256	32	0.25	0.008	0.12	0.25
122	Sputum	C	2	0.03	0.06	256	32	0.25	0.015	0.12	0.5
123	Sputum	C	2	0.015	0.06	128	32	0.12	0.015	0.06	0.25
124	Sputum	C	2	0.015	0.015	256	32	0.12	0.008	0.03	0.5
125	Sputum	C	2	0.015	0.03	256	32	0.25	0.008	0.12	0.5

126	Sputum	C	1	0.03	0.06	256	32	0.25	0.015	0.12	0.5
127	Sputum	C	2	0.015	0.06	128	16	0.12	0.008	0.03	0.25
128	Sputum	I (lung)	2	0.015	0.06	128	32	0.12	0.008	0.06	0.5
129#	Pus from lung	I (lung)	2	0.015	0.03	>256	16	>16	0.015	1	8
130	Sputum	I (lung)	1	0.015	0.008	>256	4	0.25	0.008	0.12	0.5
131	Sputum	C	1	0.015	0.03	>256	4	0.25	0.015	0.06	0.25
132	Sputum	C	2	0.015	0.03	>256	32	0.25	0.008	0.12	0.5
133	Sputum	C	2	0.015	0.06	128	64	0.25	0.015	0.06	0.25
134	Sputum	C	2	0.015	0.06	256	64	0.25	0.008	0.12	0.5
135	Sputum	C	0.5	0.015	0.03	>256	4	0.25	0.008	0.12	0.25
136	Sputum	C	1	0.015	0.03	256	32	0.06	0.008	0.03	0.25
137	Sputum	C	1	0.015	0.015	>256	64	0.25	0.008	0.12	0.5
138	Sputum	C	0.5	0.015	0.03	128	2	0.12	0.008	0.06	0.25
139	Sputum	I (lung)	0.5	0.015	0.015	256	2	0.25	0.008	0.06	0.25
140	Sputum	C	1	0.015	0.015	>256	64	0.5	0.008	0.25	0.5
141	Sputum	C	2	0.015	0.015	128	64	0.12	0.008	0.03	0.25
142	Sputum	C	1	0.03	0.12	>256	32	0.5	0.015	0.25	0.25
143	Sputum	C	2	0.06	0.06	256	64	0.5	0.03	0.25	0.5
144	Sputum	C	2	0.03	0.12	>256	64	0.5	0.03	0.25	0.5
145	Sputum	C	2	0.06	0.12	>256	32	0.5	0.03	0.12	0.25
146	Soft tissue	I (soft tissue)	1	0.03	0.12	>256	64	0.5	0.03	0.25	0.25
147	Sputum	I (lung)	1	0.03	0.12	256	8	0.25	0.015	0.12	0.25
148	Sputum	C	1	0.03	0.12	>256	64	0.25	0.015	0.12	0.5
Veterinary isolates											
149	Canine, sinus tissue	C	2	0.03	0.03	>256	32	0.12	0.015	0.03	0.5

150	Canine, sinus tissue	C	1	0.015	0.03	>256	32	0.5	0.015	0.25	0.5
151	Canine, sinus tissue	C	1	0.03	0.03	256	64	0.03	0.015	0.015	0.25
152	Feline, sinus tissue	C	1	0.015	0.06	>256	32	0.12	0.015	0.03	0.25
153	Avian, lung	C	1	0.03	0.06	>256	32	0.25	0.03	0.06	0.25
154	Avian, air sac	I (respiratory)	1	0.03	0.03	>256	64	0.12	0.015	0.03	0.5
155	Avian, coelom tissue	C	0.5	0.015	0.015	256	8	0.25	0.015	0.12	0.25
156	Avian, lung	I (lung)	1	0.03	0.06	>256	64	0.06	0.015	0.015	0.25
157	Avian, pericardium	I	2	0.03	0.06	256	64	0.12	0.03	0.06	0.25
158†	Avian, air sac	I (respiratory)	2	0.015	0.06	>256	64	0.06	0.015	0.03	0.5
159	Avian, ocular tissue	I (disseminated)	1	0.015	0.03	>256	>64	0.12	0.008	0.06	0.5
160	Avian, conjunctival swab	I (disseminated)	-	-	-	-	-	-	-	-	-
161	Avian, lung	I (lung)	-	-	-	-	-	-	-	-	-
162	Avian, lung	I (disseminated)	-	-	-	-	-	-	-	-	-

163	Avian, air sac	I (disseminated)	-	-	-	-	-	-	-	-	-
164	Avian, kidney	I (disseminated)	-	-	-	-	-	-	-	-	-
165	Avian, lung	I (lung)	-	-	-	-	-	-	-	-	-
166	Avian, air sac	I (respiratory)	-	-	-	-	-	-	-	-	-
167	Feline, nasal plaque	C	-	-	-	-	-	-	-	-	-
168	Canine, nasal plaque	C	-	-	-	-	-	-	-	-	-
169	Canine, sinus plaque	C	-	-	-	-	-	-	-	-	-

Abbreviations: AMB, amphotericin B; ANF, anidulafungin; BAL, bronchoalveolar lavage; CAS, caspofungin; C, colonisation; FLC, fluconazole; 5FC, 5-flucytosine-C; I, invasive; ITC, itraconazole; MIF, micafungin; POS, posaconazole; VRC, voriconazole.

* Disease state: For the purposes of this study human and animal patients were assigned as having invasive disease if fungal hyphae with evidence of invasion were seen on histological sections of affected tissue or body sties as well as being cultured, or were isolated from a normally sterile body site e.g. eye; or who had host risk factors, a positive *A. fumigatus* culture and radiological evidence consistent with invasive fungal disease.[13] Colonisation was defined as the isolation of *A. fumigatus* from non-sterile sites, and in human patients, where there was no clinical radiological evidence of infection.

^ Minimum effective concentrations (MECs) are reported for echinocandin drugs, anidulafungin, caspofungin and micafungin.

azole –resistant isolates.

† isolates that had discordant results of VIPcheck™ testing and Sensititre™ antifungal susceptibility testing

Table S2**.** Isolate details for 185 environmental isolates from azole-exposed (n=64) and azole naïve (n=121) sources. Sensititre™ YeastOne YO10 assay (TREK Diagnostics, USA) MIC/ minimum effective concentration (for echinocandins) values are provided for isolates tested (n=41).

Isolate No	**Jurisdiction**	**Azole status (exposed/naïve)**	**DMI fungicide used**	**Substrate**	**MIC/ MEC^ values (mg/L)**								
					AMB	**ANI**	**CAS**	**FLU**	**5-FC**	**ITC**	**MIF**	**POS**	**VRC**
1	NSW	naïve	-	soil, viticulture	1	0.03	0.06	>256	32	0.12	0.03	0.03	0.25
2	WA	exposed	myclobutanil	soil, viticulture	1	0.015	0.06	256	32	0.06	0.015	0.015	0.25
3	WA	naïve	-	soil, viticulture	2	0.03	0.03	256	2	0.12	0.015	0.03	0.25
4	NSW	naïve	-	soil, viticulture	1	0.03	0.06	256	16	0.25	0.015	0.12	0.25
5	NSW	naïve	-	soil, viticulture	1	0.015	0.03	>256	4	0.12	0.015	0.03	0.5
6	NSW	naïve	-	soil, viticulture	1	0.03	0.03	>256	16	0.12	0.03	0.06	0.25
7	NSW	naïve	-	soil, viticulture	1	0.03	0.06	256	16	0.25	0.03	0.06	0.25
8	NSW	naïve	-	soil, fig crop	1	0.015	0.03	>256	32	0.06	0.008	0.015	0.5
9	NSW	naïve	-	soil, hospital grounds	1	0.03	0.06	256	32	0.25	0.015	0.06	0.25
10	NSW	naïve	-	soil, hospital grounds	1	0.015	0.03	>256	32	0.03	0.015	0.015	0.5

11	NSW	naïve	-	soil, hospital grounds	1	0.015	0.03	>256	64	0.25	0.008	0.06	0.5
12	NSW	exposed	cyproconazole	soil, wheat crop	1	0.03	0.06	256	16	0.25	0.015	0.12	0.25
13	NSW	exposed	cyproconazole	soil, wheat crop	1	0.06	0.06	256	8	0.25	0.015	0.12	0.25
14	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.015	0.03	256	8	0.25	0.015	0.12	0.25
15	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.03	0.06	256	8	0.25	0.015	0.12	0.25
16	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.03	0.06	256	16	0.25	0.015	0.12	0.25
17	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.015	0.06	>256	64	0.06	0.015	0.015	0.25
18	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.03	0.06	256	16	0.25	0.015	0.12	0.25
19	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.03	0.03	256	32	0.03	0.015	0.015	0.25
20	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.015	0.06	256	4	0.25	0.015	0.12	0.25
21	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.03	0.06	>256	>64	0.5	0.015	0.25	0.25
22	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.015	0.015	256	64	0.06	0.008	0.03	0.5
23	TAS	exposed	tebuconazole, epoxiconazole	soil, cereal crop	1	0.03	0.03	256	4	0.25	0.03	0.12	0.25
24	WA	exposed	myclobutanil	soil, viticulture	1	0.015	0.06	256	2	0.25	0.008	0.12	0.25
25	WA	exposed	myclobutanil	soil, viticulture	2	0.03	0.06	256	64	0.25	0.015	0.12	0.25

26	WA	exposed	myclobutanil	soil, viticulture	2	0.015	0.03	256	64	0.03	0.015	0.015	0.25
27	WA	naïve	-	soil, viticulture	1	0.06	0.06	256	2	0.25	0.03	0.12	0.25
28	WA	naïve	-	soil, viticulture	1	0.015	0.03	256	32	0.03	0.008	0.015	0.25
29	WA	exposed	tebuconazole, penconazole	soil, viticulture	0.5	0.03	0.03	256	4	0.25	0.015	0.12	0.25
30	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	2	0.015	0.03	256	64	0.03	0.015	0.015	0.25
31	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	1	0.03	0.06	128	2	0.25	0.015	0.12	0.12
32†	WA	exposed	tebuconazole, penconazole	soil, viticulture	2	0.015	0.03	>256	>64	0.06	0.015	0.03	0.25
33†	WA	exposed	tebuconazole, myclobutanil	soil, viticulture	2	0.015	0.03	>256	>64	0.06	0.015	0.03	0.25
34†	NSW	exposed	cyproconazole	soil, wheat crop	2	0.015	0.06	>256	64	0.12	0.015	0.06	1
35†	WA	exposed	tebuconazole, penconazole	soil, viticulture	2	0.015	0.015	>256	>64	0.06	0.008	0.03	0.5
36	NSW	naïve	-	air, hospital indoors	2	0.015	0.03	256	16	0.12	0.008	0.06	0.5
37	NSW	naïve	-	air, hospital indoors	2	0.015	0.03	256	16	0.12	0.008	0.03	0.25
38	NSW	naïve	-	air, hospital indoors	2	0.015	0.06	256	16	0.25	0.015	0.06	0.25
39	NSW	naïve	-	air, hospital indoors	2	0.015	0.03	256	32	0.12	0.008	0.03	0.25

40	NSW	naïve	-	air, hospital indoors	2	0.015	0.03	256	32	0.25	0.015	0.06	0.25
41	NSW	naïve	-	air, hospital indoors	2	0.015	0.06	128	32	0.12	0.008	0.06	0.5
42	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
43	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
44	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
45	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
46	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
47	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
48	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
49	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
50	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
51	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
52	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
53	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
54	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-

55	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
56	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
57	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
58	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
59	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
60	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
61	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
62	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
63	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
64	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
65	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
66	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
67	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
68	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
69	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-

70	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
71	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
72	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
73	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
74	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
75	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
76	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
77	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
78	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
79	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
80	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
81	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
82	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
83	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
84	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-

85	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
86	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
87	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
88	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
89	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
90	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
91	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
92	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
93	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
94	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
95	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
96	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
97	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
98	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
99	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-

100	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
101	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
102	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
103	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
104	NSW	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
105	NSW	naïve	-	soil, figs	-	-	-	-	-	-	-	-	-
106	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
107	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
108	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
109	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
110	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
111	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
112	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-

113	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
114	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
115	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
116	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
117	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
118	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
119	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
120	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
121	NSW	naïve	-	soil, hospital grounds	-	-	-	-	-	-	-	-	-
122	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
123	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
124	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-

125	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
126	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
127	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
128	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
129	WA	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
130	WA	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
131	WA	naïve	-	soil, viticulture	-	-	-	-	-	-	-	-	-
132	WA	exposed	tebuconazole, penconazole	soil, viticulture	-	-	-	-	-	-	-	-	-
133	WA	exposed	tebuconazole, penconazole	soil, viticulture	-	-	-	-	-	-	-	-	-
134	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
135	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
136	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
137	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
138	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
139	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-

140	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
141	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
142	WA	exposed	myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
143	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
144	NSW	exposed	cyproconazole	soil, wheat crop	-	-	-	-	-	-	-	-	-
145	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
146	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
147	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
148	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
149	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
150	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
151	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
152	NSW	exposed	cyproconazole	soil, wheat crop	-	-	-	-	-	-	-	-	-
153	NSW	exposed	cyproconazole	soil, oats crop	-	-	-	-	-	-	-	-	-
154	WA	exposed	tebuconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-

155	NSW	exposed	cyproconazole	soil, wheat crop	-	-	-	-	-	-	-	-	-
156	NSW	exposed	cyproconazole	soil, wheat crop	-	-	-	-	-	-	-	-	-
157	NSW	exposed	cyproconazole	soil, wheat crop	-	-	-	-	-	-	-	-	-
158	NSW	exposed	cyproconazole	soil, wheat crop	-	-	-	-	-	-	-	-	-
159	WA	exposed	tebuconazole, penconazole	soil, viticulture	-	-	-	-	-	-	-	-	-
160	WA	exposed	tebuconazole, penconazole	soil, viticulture	-	-	-	-	-	-	-	-	-
161	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
162	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
163	WA	exposed	tebuconazole, penconazole	soil, viticulture	-	-	-	-	-	-	-	-	-
164	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
165	WA	exposed	tetraconazole, myclobutanil	soil, viticulture	-	-	-	-	-	-	-	-	-
166*	VIC	naïve	-	air, nature reserve	-	-	-	-	-	-	-	-	-
167*	VIC	naïve	-	air, nature reserve	-	-	-	-	-	-	-	-	-
168*	VIC	naïve	-	soil, domestic garden	-	-	-	-	-	-	-	-	-
169*	VIC	naïve	-	air, nature reserve	-	-	-	-	-	-	-	-	-

170*	VIC	naïve	-	soil, domestic garden	-	-	-	-	-	-	-	-	-
171*	VIC	naïve	-	air, nature reserve	-	-	-	-	-	-	-	-	-
172*	VIC	naïve	-	soil, domestic garden	-	-	-	-	-	-	-	-	-
173*	VIC	naïve	-	soil, domestic garden	-	-	-	-	-	-	-	-	-
174*	VIC	naïve	-	air, domestic garden	-	-	-	-	-	-	-	-	-
175*	VIC	naïve	-	air, domestic barn	-	-	-	-	-	-	-	-	-
176*	VIC	naïve	-	air, domestic garden	-	-	-	-	-	-	-	-	-
177*	VIC	naïve	-	air, nature reserve	-	-	-	-	-	-	-	-	-
178*	VIC	naïve	-	air, nature reserve	-	-	-	-	-	-	-	-	-
179*	VIC	naïve	-	air, domestic barn	-	-	-	-	-	-	-	-	-
180*	VIC	naïve	-	soil, domestic garden	-	-	-	-	-	-	-	-	-

181*	VIC	naïve	-	soil, domestic garden	-	-	-	-	-	-	-	-	-
182*	VIC	naïve	-	air, domestic indoor	-	-	-	-	-	-	-	-	-
183*	VIC	naïve	-	air, nature reserve	-	-	-	-	-	-	-	-	-
184*	VIC	naïve	-	air, nature reserve	-	-	-	-	-	-	-	-	-
185*	VIC	naïve	-	soil, domestic garden	-	-	-	-	-	-	-	-	-

Abbreviations: AMB, amphotericin B; ANF, anidulafungin; CAS, caspofungin; FLC, fluconazole; ITC, itraconazole; MIF, micafungin; MEC, minimum effective concentration; NA, not available; POS, posaconazole; VRC, voriconazole.

^ Minimum effective concentrations (MECs) are reported for echinocandin drugs, including anidulafungin, caspofungin and micafungin.

† isolates that had discordant results of VIPcheck™ testing and Sensititre™ antifungal susceptibility testing.

* isolates obtained from culture collection (Talbot JJ, Houbraken J, Frisvad C et al. Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for animal and human fungal pathogens. *PLoS ONE* 2017; 12: e0181660).

6.4 Conclusions

This large study showed a low prevalence of azole resistance amongst *A. fumigatus* clinical and environmental isolates in Australia (2.0%). Azole resistance was only detected amongst human patients, not environmental and veterinary isolates. A low prevalence is welcome news in an antimicrobial resistance era, where azole resistance has spread globally with high prevalence reported out of some regions, including up to 26% in human (van der Linden et al., 2015) and 91% in environmental isolates (Zhang et al., 2017) in western Europe.

The azole resistant isolates in this study had *cyp51A* mutations associated with environmental development (TR_{34}/L98H and G54). If environmental evidence of ARAf was confirmed in Australia, whole genome sequencing could help determine the relatedness between clinical isolates to determine the effects of DMI fungicide use. Although ARAf prevalence was low in this study, continued surveillance is recommended for monitoring and selection of treatment regimens.

This study also demonstrated the usefulness of heat treatment for recovering isolates of *A. fumigatus* from soil (method 2) (Nováková et al., 2014) (Houbraken and Samson, 2006) which targets the activation of ascospores and was particularly useful for isolating *A. fumigatus* from azole-exposed soils.

The *cyp51A A. fumigatus* homology model highlights the usefulness of such models for predicting the significance of *cyp51A* mutation; their potential to interfere with azole drug binding to the protein and complex stability. This model was based on the most recently published *A. fumigatus cyp51B* model (Hargrove et al., 2015). Thus this research also further highlights the usefulness of the *A. fumigatus cyp51B* as a homology model for the crystal structure of *A. fumigatus cyp51A* by demonstrating how the G54R mutation prevents docking of large azole molecules but permits docking of smaller molecules to the *cyp51A* haem complex, leading to resistance to voriconazole and susceptibility to itraconazole and posaconazole.

A selection of full genome sequences from this study will undergo further examination by Professor Matthew Fisher's group at the Imperial College London to determine their genetic relatedness amongst global populations of *A. fumigatus* isolates.

Interestingly, members of the *A. viridinutans* species complex (AVSC) causing clinical disease have high azole MICs (Balajee et al., 2006, Alcazar-Fuoli et al., 2008, Vinh et al., 2009, Posteraro et al., 2011, Coelho et al., 2011, Gyotoku et al., 2012, Barrs et al., 2013, Peláez et al., 2013, Sugui et al., 2014, Alvarez-Pérez et al., 2014); however the *cyp51A* gene has not been sequenced for these species, so it is unknown if their resistance is inherent or acquired and possibly attributed to *cyp51A* mutations as in ARAf isolates. This will be further examined in Chapter 7.

6.5 References

ALCAZAR-FUOLI, L., MELLADO, E., ALASTRUEY-IZQUIERDO, A., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2008. *Aspergillus* section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. *Antimicrobial Agents and Chemotherapy,* 52**,** 1244-1251.

ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., BLANCO, J. L., GARCIA, M. E., KWON, M., MUÑOZ, P., CUENCA-ESTRELLA, M., BOUZA, E. & PELÁEZ, T. 2014. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. *Journal of Medical Microbiology,* 63**,** 617-619.

ARENDRUP, M. C., VERWEIJ, P. E., MOUTON, J. W., LAGROU, K. & MELETIADIS, J. 2017. Multicentre validation of 4-well azole agar plates as a screening

method for detection of clinically relevant azole-resistant

Aspergillus fumigatus. *Journal of Antimicrobial Chemotherapy,* 72**,** 3325-3333.

BALAJEE, S. A., NICKLE, D., VARGA, J. & MARR, K. A. 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryotic Cell,* 5**,** 1705-1712.

BARRS, V. R., VAN DOORN, T. M., HOUBRAKEN, J., KIDD, S. E., MARTIN, P., PINHEIRO, M. D., RICHARDSON, M., VARGA, J. & SAMSON, R. A. 2013. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One,* 8**,** e64871.

CHEN, J., LI, H., LI, R., BU, D. & WAN, Z. 2005. Mutations in the cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *Journal of Antimicrobial Chemotherapy,* 55**,** 31-37.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) 2018. M59 Epidemiology cutoff values of antifungal susceptibility testing Clinical and Laboratory Standards Institute: 2nd ed. CLSI supplement M59, Wayne, PA.

COELHO, D., SILVA, S., VALE-SILVA, L., GOMES, H., PINTO, E., SARMENTO, A. & PINHEIRO, M. D. 2011. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. *Medical Mycology,* 49**,** 755-759.

ESPINELL-INGROFF, A., DIEKEMA, D. J., FOTHERGILL, A., JOHNSON, E., PELAEZ, T., PFALLER, M. A., RINALDI, M. G., CANTON, E. & TURNIDGE, J. 2010. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Journal of Clinical Microbiology,* 48**,** 3251-3257.

GLASS, N. L. & DONALDSON, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology,* 61**,** 1323-1330.

GYOTOKU, H., IZUMIKAWA, K., IKEDA, H., TAKAZONO, T., MORINAGA, Y., NAKAMURA, S., IMAMURA, Y., NISHINO, T., MIYAZAKI, T., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., YASUOKA, A., YAGUCHI, T., OHNO, H.,

MIYZAKI, Y., KAMEI, K., KANDA, T. & KOHNO, S. 2012. A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. *Med Mycol.,* 50**,** 631-636.

HARGROVE, T. Y., WAWRZAK, Z., LAMB, D. C., GUENGERICH, F. P. & LEPESHEVA, G. I. 2015. Structure-functional characterization of cytochrome P450 sterol 14α-demethylase (*CYP51B*) from *Aspergillus fumigatus* and molecular basis for the development of antifungal drugs. *The Journal of Biological Chemistry* 290**,** 23916-23934.

HOUBRAKEN, J. & SAMSON, R. A. 2006. Standardization of methods for detecting heat-resistant fungi. *Advances in Experimental Medicine and Biology,* 571.

KIDD, S. E., GOEMAN, E., MEIS, J. F., SLAVIN, M. A. & VERWEIJ, P. E. 2015. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses,* 58**,** 350-355.

LIU, M., ZHENG, N., LI, D., ZHENG, H., ZHANG, L., GE, H. & LIU, W. 2016. cyp51A-based mechanism of azole resistance in *Aspergillus fumigatus*: Illustration by a new 3D Structural Model of *Aspergillus fumigatus CYP51A* protein. *Medical Mycology,* 54**,** 400-408.

NASH, A. & RHODES, J. 2017. Simulations of *CYP51A* from *Aspergillus fumigatus* in a model bilayer provide insights into triazole drug resistance. *Medical Mycology,* myx056**,** 1-13.

NOVÁKOVÁ, A., HUBKA, V., DUDOVÁ, Z., MATSUZAWA, T., KUBÁTOVÁ, A., YAGUCHI, T. & KOLAŘÍK, M. 2014. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. *Fungal Diversity.*, 64**,** 253-274.

PELÁEZ, T., ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., VALERIO, M., BLANCO, J. L., GARCIA, M. E., MUÑOZ, P., CUENCA-ESTRELLA, M. & BOUZA, E. 2013. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. *Journal of Medical Microbiology,* 62**,** 474-478.

POSTERARO, B., MATTEI, R., TRIVELLA, F., MAFFEI, A., TORRE, A., DE CAROLIS, E., POSTERARO, P., FADDA, G. & SANGUINETTI, M. 2011. Uncommon *Neosartorya*

udagawae fungus as a causative agent of severe corneal infection. *J Clin Microbiol,* 49**,** 2357-2360.

SAMSON, R. A., HOUBRAKEN, J., THRANE, U. & FRISVAD, J. C. 2010. *Food and Indoor fungi,* Utrecht, Centraalbureau voor Schimmelcultures.

SNELDERS, E., HUIS IN 'T VELD, R. A., RIJS, A. J. M., KEMA, G. H. J., MELCHERS, W. J. G. & VERWEIJ, P. E. 2009. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazole. *Applied and Environmental Microbiology,* 75**,** 4053-4057.

SUGUI, J. A., PETERSON, S. W., FIGAT, A., HANSEN, B., SAMSON, R. A., MELLADO, E., CUENCA-ESTRELLA, M. & KWON-CHUNG, K. J. 2014. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J Clin Microbiol,* 52**,** 3707-3721.

VAN DER LINDEN, J. W., ARENDRUP, M. C., WARRIS, A., LAGROU, K., PELLOUX, H., HAUSER, P. M., CHRYSSANTHOU, E., MELLADO, E., KIDD, S. E., TORTORANO, A. M., DANNAOUI, E., GAUSTAD, P., BADDLEY, J. W., UEKOTTER, A., LASS-FLORL, C., KLIMKO, N., MOORE, C. B., DENNING, D. W., PASQUALOTTO, A. C., KIBBLER, C., ARIKAN-AKDAGLI, S., ANDES, D., MELETIADIS, J., NAUMIUK, L., NUCCI, M., MELCHERS, W. J. & VERWEIJ, P. E. 2015. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerging Infectious Diseases,* 21**,** 1041-4.

VAN DER LINDEN, J. W., CAMPS, S. M., KAMPINGA, G. A., ARENDS, J. P., DEBETS-OSSENKOPP, Y. J., HAAS, P. J., RIJNDERS, B. J., KUIJPER, E. J., VAN TIEL, F. H., VARGA, J., KARAWAJCZYK, A., ZOLL, J., MELCHERS, W. J. & VERWEIJ, P. E. 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clinical Infectious Diseases,* 57**,** 513-20.

VINH, D. C., SHEA, Y. R., SUGUI, J. A., PARRILLA-CASTELLAR, E. R., FREEMAN, A. F., CAMPBELL, J. W., PITTALUGA, S., JONES, P. A., ZELAZNY, A., KLEINER, D., KWON-CHUNG, K. J. & HOLLAND, S. M. 2009. Invasive aspergillosis due to *Neosartorya udagawae*. *Clinical Infectious Diseases,* 49**,** 102-111.

ZHANG, J., SNELDERS, E., ZWAAN, B. J., SCHOUSTRA, S. E., MEIS, J. F., VAN DIJK, K., HAGEN, F., VAN DER BEEK, M. T., KAMPINGA, G. A., ZOLL, J., MELCHERS, W. J. G., VERWEIJ, P. E. & DEBETSA, A. J. M. 2017. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *mBio,* 8**,** e00791-17.

Chapter 7. Antifungal susceptibility testing and *cyp51A* analysis of *Aspergillus viridinutans* species complex isolates of environmental and clinical origin.

7.1 Introduction

Reports of aspergillosis due to *Aspergillus viridinutans* species complex (AVSC) generally describe high minimum inhibitory concentration (MIC) of azole antifungals compared to MICs for wild-type *A. fumigatus* (Balajee et al., 2006, Alcazar- Fuoli et al., 2008, Vinh et al., 2009, Posteraro et al., 2011, Coelho et al., 2011, Gyotoku et al., 2012, Barrs et al., 2013, Peláez et al., 2013, Sugui et al., 2014, Alvarez-Pérez et al., 2014). It is assumed this is an inherent feature of these complex members; however no studies have investigated the possibility of *cyp51A* mutations, the main cause of azole resistance in other *Aspergillus* species (*A. fumigatus*). This chapter investigates antifungal susceptibility testing and sequencing of the *cyp51A* gene in clinical and environmental AVSC isolates.

7.2 Premise

Azole therapeutics are the first line of defence when treating aspergillosis. Azoles bind to the active site of the *cyp51A* protein, preventing demethylation of lanosterol to ergosterol, required for maintaining fungal cell walls (Hargrove et al., 2015). Alterations to this gene (tandem repeats and single point mutations) are the main cause of azole resistance in *A. fumigatus* isolates, generally identified *in vitro* via microbroth dilution antifungal susceptibility testing and sequencing of the *cyp51A* gene.

This research aimed to characterise and determine the prevalence of *cyp51A* mutations amongst AVSC members to establish if these are a minor or major

mechanism of azole resistance.

I performed *cyp51A* sequencing under the supervision of Dr Jos Houbraken at the Westerdijk Institute, Utrecht, the Netherlands, while undertaking my Australian Government Endeavour Research Fellowship. Primers were based on published primers for the *cyp51A* gene in *A. fumigatus* species (Chen et al., 2005). Degenerate primer sets were developed by searching for the *cyp51A* region in the *A. udagawae* whole genome sequence (Kusuya et al., 2015). Sequencing included all AVSC isolates available in the Westerdijk Institute collection and those described in Chapter 3, including at least one representative isolate from every species in the AVSC. Following PCR optimisation, Sanger sequencing was performed in-house. The FunResDB online database was used to determine mutation positions and for comparison with wild-type *A. fumigatus cyp51A* sequences (Weber et al., 2017).

Antifungal susceptibility testing initially used the testing kit Sensititre™ YeastOneYO8 system (TREK Diagnostic Systems Ltd., OH, USA) (n=48), with MIC, or minimum effective concentration (MEC) values (for echinocandins) determined as published (Talbot et al., 2015, Arendrup et al., 2017). As no ECVs have been established for the AVSC, the end-point for determining MIC/MEC values was read at 72 hours, and azole MIC value interpretation was based on epidemiological cut-off values for *A. fumigatus* (for itraconazole and voriconazole, a MIC of <1 mg/L was WT and ≥2 mg/L was non-WT (NWT); for posaconazole, a MIC of ≤0.25 mg/L was WT, and of >0.5 mg/L was NWT) (Espinell- Ingroff et al., 2010, Clinical and Laboratory Standards Institute (CLSI), 2018). Forty-eight isolates sporulated adequately for Sensititre™ testing (see Supplementary Table 3). Of these, 38 (79%) demonstrated non-WT MICs to at least one azole drug, including 10 (21%) to itraconazole (≥2 mg/L), 37 (77 %) to voriconazole (≥2 mg/L) and 7 (15%) to posaconazole (>0.5 mg/L). Five (10%; 2 clinical, 3 environmental) isolates demonstrated non-susceptibility to itraconazole (>16 μg/mL). Twenty isolates had MICs at or above the epidemiological cut-off value, but were not non-WT, including nine (19%) to itraconazole, 11 (23%) to posaconazole and four (8 %) to voriconazole.

Unexpectedly only five isolates demonstrated resistance to itraconazole. Shortly after this, Prof Vanessa Barrs shared the results of an international study on antifungal susceptibility testing in the AVSC with me prior to publication. In their study (later published as Lyskova et al. 2018), comparing Sensititre™ testing and EUCAST antifungal susceptibility testing (Arendrup et al., 2017) revealed a discrepancy between the two for AVSC isolates (Lyskova et al. 2018). Thus, I further investigated my results with another globally accepted technique, the Clinical Laboratory Standards Institute (CLSI) method of antifungal susceptibility testing (Clinical Laboartory Standards Institute (CLSI), 2017). For moulds, CLSI and EUCAST testing produces comparable MICs (Mirchevska and Bosshard, 2012). Australian mycology reference laboratories routinely use the Sensititre™ method. Although this involves the same sample spore density as the CLSI method, the microbroth dilution technique differs. Thus the testing must be external which my colleagues in the Netherlands, Dr Jacques Meis (Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital (CWZ), Nijmegen, the Netherlands and Center of Expertise in Mycology Radboudumc/CWZ, Nijmegen, the Netherlands) and Dr Ferry Hagen (Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital (CWZ), Nijmegen, the Netherlands and Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands) arranged at their national *Aspergillus* reference laboratory. They obtained data from 37/56 isolates that sporulated enough to satisfy testing requirements, returning strikingly different results compared to my Sensititre™ testing, with many more isolates returning high MICs or non-susceptibility azole phenotypes. This was consistent with the findings of Lyskova et al. (2018) who reported an overall agreement between EUCAST and Sensititre™ of 67% when Sensititre™ plates were read at 48 hours and 82% at 72 hours (Lyskova et al., 2018). Comparing my Sensititre™ results to those obtained via CLSI for 31 isolates tested using both methods, concordant results for detecting high and low MICs were 55% for itraconazole, and 87% for voriconazole and posaconazole. These results are also consistent with the Lyskova *et al.* (2018) findings of a lower agreement between EUCAST and Sensititre™ for itraconazole, with a much lower detection rate for resistance or high MIC to itraconazole detection using the Sensititre™ method (Lyskova et al., 2018).

While performing CLSI testing, Dr Meis and Dr Hagen also tested the 37 AVSC isolates against luliconazole (an imidazole), currently being tested as an alternative first-line triazole for aspergillosis, showing promise for treating azole resistant *Aspergillus* isolates (Abastabar et al., 2016).

To further investigate the significance of the *cyp51A* mutations sequenced, homology was modelled in collaboration with Prof David Hibbs and Ms Felcia Lai (Faculty of Pharmacy, the University of Sydney). These models were based on published homology models for *A. fumigatus cyp51A* with the closest percentage match (*Saccharomyces cerevisiae cyp51A*) (Liu et al., 2016). Additional AVSC models were developed using the amino acid profiles from a selection of *cyp51A* sequences amplified in this study, to compare and determine the significance of susceptible and non-susceptible isolate profiles.

7.3 Unpublished Manuscript

The following manuscript has not been submitted to a journal.

Talbot J. J., Samson R. A., Kidd S. E., Meis J. F., Hagen F, Hibbs D. E., Lai F., Verweij P. E., Barrs, V. R., Houbraken J. *Cyp51A* mutations in clinical and environmental isolates of the *Aspergillus viridinutans* species complex.

Abstract

Over the past decade reports of aspergillosis in humans and other animals due to the *Aspergillus viridinutans* species complex (AVSC) have increased. Azole resistance is common among AVSC infections, carrying a poor prognosis. *Cyp51A* gene mutations are recognized as the most common cause of acquired azole resistance in *Aspergillus fumigatus* isolates. This study aimed to determine if *cyp51A* mutations in ASVC members are associated with an azole resistant phenotype through amplification of *cyp51A* amongst 56 AVSC members (clinical and environmental isolates) and antifungal susceptibility testing (CLSI [n=37]). CLSI testing detected very high minimum inhibitory concentrations (MICs) to azoles in 84% (31/37) of isolates. MICs for the imidazole antifungal luliconazole were low for all isolates. *Cyp51A* was amplified using novel degenerate primers, and sequences compared

to *A. fumigatus*, with a total of 113 non-synonymous mutations. Three *cyp51A* mutations (M172A, M172V and D255G) previously reported in association with azole resistance in *A. fumigatus* were common amongst all AVSC members (high and low MICs). Two environmental isolates with non-susceptibility to itraconazole at the highest drug concentration, and high MICs to voriconazole and isavuconazole, harboured the G138C mutation, previously associated with azole resistance in *A. fumigatus*. Some mutations were only identified amongst AVSC members with high azole MICs; however no other known acquired *cyp51A* alterations reported for *A. fumigatus* were identified. *Cyp51A* homology modelling did not change the overall protein structure significantly to confer resistance. *Cyp51A* mutations are not a major mechanism of antifungal resistance among AVSC isolates. The imidazole antifungal luliconazole shows promise for treating infections caused by azole-resistant AVSC.

Introduction

Aspergillosis, usually due to *Aspergillus fumigatus*, can cause invasive fatal disease. Triazole antifungal drugs target fungal ergosterol synthesis (itraconazole, voriconazole, posaconazole and more recently isavuconazole) and are the mainstay of treatment (Ullmann et al., 2018). Since the late 1990s aspergillosis treatment in humans has been complicated by azole resistance, most commonly reported in *A. fumigatus* isolates (Verweij et al., 2016). Poor clinical outcomes are also associated with increased reports of infection by other species in *Aspergillus* section *Fumigati* with decreased antifungal susceptibility *in vitro* and *in vivo*, including members of the *Aspergillus viridinutans* species complex (AVSC) (Talbot and Barrs, 2018).

Azole drug resistance can be innate or acquired (van der Linden et al., 2011). Acquired resistance in *A. fumigatus* clinical and environmental isolates is mostly associated with tandem repeats in the promoter region and single point mutations of the *cyp51A* gene. Acquired antifungal resistance was first reported in isolates from human patients in the United States (Denning et al., 1997), then the Netherlands where it was associated with the use of triazole fungicides in agriculture (Chowdhary et al., 2013). Azole resistance due to *cyp51A* mutations has since been reported globally among clinical and environmental isolates (Chowdhary et al., 2017), reinforcing the concept of a one-health approach also in fungal diseases (Talbot and Barrs, 2018, Chowdhary and Meis, 2018).

More than 50 mutations and tandem repeat combinations in the *cyp51A* gene have been

detected among *A. fumigatus* isolates (Weber et al., 2017). TR_{34}/L98H and TR_{46}/Y121F/T289 are most frequently associated with environmentally acquired azole resistance (van der Linden et al., 2013), while other mutations have been associated with therapeutic azole drugs (G54, G138, G448, M220) (Howard and Arendrup, 2011).

The AVSC contains nine species (*A. arcoverdensis, A. aureolus, A. felis, A. frankstonensis, A. pseudoviridinutans, A. siamensis, A. udagawae, A. viridinutans, A. wyomingensis*). In 2014, *A. parafelis* and *A. pseudofelis* were described as novel species closely related to *A. felis*; however, analysis of more strains indicates they are *A. felis* (Talbot JJ et al., 2017, Lyskova et al., 2018, Hubka et al., 2018). Since 2005, *A. felis, A. pseudoviridinutans, A. udagawae,* and *A. wyomingensis,* have been increasingly associated with localized or disseminated infections that are difficult to treat and often have high minimum inhibitory concentrations (MICs) of triazole antifungals (Talbot and Barrs, 2018). Innate resistance is thought to be responsible for the high intrinsic resistance among these species (van der Linden et al., 2011). However, whether mutations of the *cyp51A* gene could be involved has not been investigated.

This study investigated correlations between azole resistant phenotype and *cyp51A* genotype among clinical and environmental AVSC isolates (n=56, see Supplementary Table 1) by amplifying the *cyp51A* gene, antifungal susceptibility testing and *cyp51A* protein homology modelling.

Materials and Methods

Isolates

The 56 archived AVSC isolates retrieved from the fungal culture biobank (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands (n=55) and one from South Australia Pathology, Adelaide, South Australia included 6 human isolates, 20 animal isolates, 25 environmental isolates and 5 isolates of unknown origin (see Supplementary Table 1).

Antifungal susceptibility testing

Testing for susceptibility to azole antifungal drugs was performed using the CLSI microbroth dilution antifungal susceptibility method (n=37) with MIC, or minimum effective concentration (MEC) values (for echinocandins) determined as previously described (Clinical

Laboartory Standards Institute (CLSI), 2017). As clinical breakpoints have not been assigned to AVSC species, MIC interpretation did not designate isolates as susceptible or resistant but as high or low (Lyskova et al., 2018), based on epidemiological cut-off values (ECVs) for *Aspergillus fumigatus* (ECV of itraconazole, voriconazole and isavuconazole of 1 mg/L; for posaconazole 0.5 mg/L) (Espinell-Ingroff et al., 2010, Clinical and Laboratory Standards Institute (CLSI), 2018). Luliconazole MIC values were reported but not assigned as susceptible or resistant as clinical breakpoints have not been determined for *Aspergillus* species.

Geometric mean values, range, MIC_{50} and MIC_{90} values were calculated in Excel. Standard deviations and t-scores were calculated to determine if triazole MICs for clinical isolates were higher than those of environmental isolates with significance set at $P<0.05$ using a two- tailed test (data not shown).

Cyp51A gene targeting

DNA was extracted from all isolates using the MoBio DNA isolation kit (QIAGEN Pty Ltd, Chadstone Centre, Australia and Venlo, the Netherlands). Multiple primer pairs were designed to amplify the *cyp51A* gene.

Primer design was based on 3 primer pairs of *cyp51A* gene primer pairs developed by Chen et al 2005: CYP1-L, CYP 1-R, CYP 2-L, CYP 2-R, CYP 3-L and CYP 3-R. Using these, degenerate primers were developed from the whole genome sequence for *A. udagawae* IFM 46973 (Kusuya et al., 2015) available on GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi.) (849273732:670000-675000, contig:Aud0040) using MEGA6 (Tamura et al., 2013). In total eight primer pairs were developed (see Table 3) and synthesized by Integrated DNA Technologies (Iowa, United States).

Conventional PCR was performed in a Life Technologies Applied Biosystems, 2720 Thermal Cycler for 5 minutes at 94°C; 35 cycles of 30 seconds at 94°C, 30 seconds at 52°C, 1 minute at 72°C; then 5 minutes at 72°C and hold at 10°C. PCR product was purified in-house in a Sensoquest Lab cycler and Sanger sequencing on a Hitachi/Applied Biosystems 3730xL Genetic Analyzer.

Sequences were analysed using Seqman Pro, DNAstar, Version 12.1.0. Sequences were aligned in MEGA v6 using MUSCLE alignment. Phylogenetic trees were constructed in

MEGA v6 using the Kimura 2 + gamma distribution, maximum likelihood discrete data method (tree searching method of 1,000 replicate trees) and bootstrapping. All sequences were submitted to GenBank (accession numbers MF178327-MF178401; see Supplementary Table 1).

Data were analysed for mutations using the FunResDB, comparing *Cyp51A* sequences of AVSC isolates with *A. fumigatus* reference strain ATCC36607 (Weber et al., 2017). Mutations in isolates with high MICs were compared to other isolates of the same species and all isolates in the complex.

Cyp51A homology modelling

A. fumigatus cyp51A sequence (GenBank accession no. AF338659) was retrieved from the universal protein resources (http:// www.uniprot.org) and a NCBI BLAST (blastp) search was performed to identify a suitable template. The crystal structure of *Sacchyromyces cerevisiae cyp51A* (PDB ID: 5EQB) was selected as the template for homology modelling as it is the *cyp51A* protein that returned the highest sequence identity in blastp search (resolution 2.19 Å, max and total score 506, query cover 94 %, identity 51 %). Five homology models were produced based on the template using Schrödinger's Prime (Jacobson et al., 2002, Jacobson et al., 2004), and the best model (model B) was selected after testing their quality with Verify 3D (84.66 % of residues scored a 3D-1D score >=0.2, see Supplementary Table 4) (Bowie et al., 1991, Luthy et al., 1992). Homology models of *A. felis* clade species *A. felis* (model F), *A. viridinutans* (model G) and *A. aureolus* (model H) were built based on model B using the *cyp51A* amino acid sequences of isolates DTO 131-G1, DTO 050-F1 and DTO 278-B7, respectively (see Supplementary Table 1 [GenBank accession numbers] and Supplementary Table 4 [Verify 3D results]). The models were verified with Verify 3D. Additionally, model F, the low azole MICs *A. felis* azole homology model, was subjected to mutations present in three *A. felis* clade isolates demonstrating high azole MICs (mutations T215S/G138C [model I] and S197C/ Q340R [model J], see Table 2 for isolate details). Finally, for models containing mutations at positions previously associated with azole resistance in *A. fumigatus* isolates (models H [V101] and I [G138]), mutations were added to *A. fumigatus* model B, producing model K (mutations from model I, G138C/T215S) and model L (mutations from model H, V101L/A103T/A234V/I360V/V428I/G505R/Q423D/F478V).

Molecular docking and Molecular Mechanics-Generalised Born Surface Area

Models F to L were prepared for molecular docking using Protein Preparation Wizard (Sastry et al., 2013). 2D structures of itraconazole, posaconazole and voriconazole were first drawn using 2D sketcher then prepared with Ligprep (Schrödinger Release 2017-3: LigPrep). Docking studies for the three triazoles with these models was carried out *via* Glide with standard prevision (SP) (Halgren et al., 2004, Friesner et al., 2004, Friesner et al., 2006) to determine the binding affinity between the ligand and the receptor. For each model, conformers of the triazoles that interact with the protein with the expected binding mode in SP study were chosen for the next step. They were subjected to minimisation *via* Macromodel (Schrödinger Release 2017-3: MacroModel), followed by ConfGen (Watts et al., 2010) to generate alternative low energy conformations. Docking study for these conformers was then carried out *via* Glide with extra precision (XP) (Halgren et al., 2004, Friesner et al., 2004, Friesner et al., 2006). Only the conformers with the best docking score in XP analysis were considered. Docking scores from Glide XP of each azole-resistant receptor-ligand interaction were compared to their azole-susceptible counterparts. Since model G and H, which are based on *A. viridinutans and A. aureolus* respectively, do not have corresponding low azole MIC models, they were also compared with model F. Results were interpreted as the more negative the docking score, the better the binding affinity. Molecular Mechanics-Generalised Born Surface Area (MMGBSA) binding free energy (ΔG(binding)), ligand strain energy and receptor strain energy of the ligand-receptor calculations were also performed using Prime (Li et al., 2011) based on XP docking studies; residues within 20 Å from the ligand were set flexible. Default settings were used for all calculations. ΔG(binding) indicated how strong the binding was between the ligand and receptor, and ligand and receptor strain energy indicated how much energy the ligand and protein required to distort themselves into the appropriate binding pose respectively.

Root-mean-square deviation scores

The differences between model F and azole-resistant models G-J were measured *via* superimposition and determination of root-mean-square deviation (RMSD) scores for comparison of structural similarity.

Results

Antifungal susceptibility testing

Clinical Laboratory Standards Institute (CLSI) antifungal susceptibilitytesting was performed to differentiate isolates with high MIC from those with low MIC phenotype.

Thirty-seven AVSC isolates sporulated adequately for antifungal susceptibility testing (Supplementary Table 2). Of these, 31 (84%) were defined as having high MICs to at least one azole, including 20 (54%) to itraconazole (>1 mg/L), 31 (84%) to voriconazole (>1 mg/L), 30 (81%) to isavuconazole (>1 mg/L) and one to posaconazole (>0.5 mg/L). Of these isolates, 15 were environmental, 14 were clinical, and two were of unknown origin. Comparisons of MIC geometric mean, MIC ranges, MIC_{50} and MIC_{90} results for environmental versus clinical AVSC isolates are presented in Table 1. Environmental isolates had higher MICs than clinical isolates but this was not statistically significant (isavuconazole, itraconazole and posaconazole *P*=>0.2; voriconazole *P*=0.1-0.2). All isolates were inhibited by luliconazole (geometric mean 0.002 mg/L, MIC_{90} 0.002 mg/L, range 0.001- 1 mg/L). Six AVSC isolates (four clinical, two environmental and one unknown) had low MICs for all azole drugs tested. Poor sporulation in 19 isolates precluded CLSI testing.

Cyp51A sequencing

A total of 204 synonymous and 113 non-synonymous mutations were identified amongst the 56 AVSC isolates (see Supplementary Table 3). Of the non-synonymous mutations, all AVSC isolates tested, regardless of azole phenotype, had the mutations W6L, V15M, K80R, D255G, C270S, I367L, H403Y and L464I. At amino acid position M172, all isolates had a mutation of either M172V (n=55) or M172A (n=1).

Isolates demonstrating high azole MICs had 15 unique mutations not present in isolates with low MICs (see Table 2). This included G138C, found in two environmental *A. felis*-clade isolates that were non-susceptible to itraconazole at the highest concentration tested, had high MICs to voriconazole and isavuconazole, with one also demonstrating MICs at the *A. fumigatus* ECV to posaconazole, and the other an intermediate MIC to posaconazole (Table 2). Both isolates also harboured a unique SNP, T215S (see Table 2). Two *A. aureolus* environmental isolates harboured the non-synonymous mutation V101L; isolate DTO 278-B7 had a MIC at the *A. fumigatus* ECV to posaconazole only, while DTO 331-G6 did not sporulate to be used in antifungal susceptibility testing. Both isolates also harboured unique *cyp51A* SNPs A103T,

A234V, I360V, V428I, Q423D, F478V, G505R (see Table 2).

A phylogenetic tree for *cyp51A* sequences groups isolates of the same species (see Figure 1).

Cyp51A homology modelling

Five AVSC *cyp51A* homology models (models F to J) compared amino acid profiles of low azole MIC value (model F *A. felis*-clade, DTO 131-G1) with high azole MIC value isolates (model G *A. viridinutans*, DTO 050-F1; model H *A. aureolus*, DTO 278-B7; model I *A. felis* clade, DTO 341-E5; model J *A. felis*-clade, DTO 131-E6) (see Figure 2). Docking scores and MMGBSA analysis results are presented in Table 4. Compared to model F, there were no significant changes to the overall protein structure to confer resistance in models G to J. For itraconazole, the most negative value docking score was with model I (-11.03 kcal/mol) and most negative ΔG(binding) value was with low azole MIC model F (-148.403 kcal/mol), with a higher potential for azole binding in model F compared to models G to J. For posaconazole's interactions the most negative docking score was with model I (-11.54 kcal/mol) and the most negative ΔG(binding) value was with model G (-148.061 kcal/mol). Results for voriconazole were inconsistent, with model H having the most negative docking score (-7.881 kcal/mol) and ΔG(binding) value (-64.09 kcal/mol) the highest potential for azole binding (see Table 4). RMSD scores demonstrated high similarity between models compared to the susceptible *A. felis* (model F), a difference of less than 0.2 Å for all models (model I= 0.2004 Å; model J= 0.1929 Å; model G=0.0613 Å; model H= 0.0570 Å). Two *A. fumigatus cyp51A* homology models (models K and L) were constructed to determine whether mutations reported in association with azole resistance in *A. fumigatus* (positions G138 and V101) altered the protein structure like the AVSC based models H and I. This confirmed the results of AVSC models H and I, with no major difference for docking and MMGBSA analysis with these mutations in the *A. fumigatus* model (see Table 4 and Supplementary Figure 1).

Discussion

There were nine *cyp51A* mutations amongst all AVSC isolates compared to *A. fumigatus* wild-type (see Supplementary Table 3). As these were found in isolates with high and low MICs, they are likely non-functional in relation to azole resistance. Also, species specific amino acid substitutions were observed. Those positions could be interesting targets for identification (either on species or complex level) or for diagnostics, and the degenerate primers developed

could have clinical application for other pathogenic, non-*A. fumigatus Aspergillus* species. Further, phylogenetic analysis of the *cyp51A* gene sequences resolved all AVSC species.

Of the *cyp51A* SNPs identified in isolates with high MICs only, none shared the same susceptibility phenotype (see Table 2). *Cyp51A* modelling was used to determine the significance of these mutations. Interactions in high azole MIC value models (model G to J) were proposed to have weaker binding affinity and less azole binding in mutated models G to J, hence these models might confer resistance. However, the resulting homology models varied. Itraconazole interactions were most consistent, with model F having the strongest binding affinity to this drug. This trend was also observed in posaconazole's interactions and voriconazole demonstrated conflicting results, most likely due to its smaller size, as it requires little energy to position itself to the correct binding mode. These results and the similarity between the protein structures demonstrated via RMSD score calculations suggests that the *cyp51A* mutations in these isolates do not significantly change the overall protein structure and thus do not directly interfere with azole drug binding to confer resistance. Future work investigating genome sequences of AVSC susceptible and resistant isolates could clarify these amino acid profile differences, potentially determining strain lineage as well as identifying other genetic causes of azole resistance. *Aspergillus felis* is heterothallic and progeny, from directed mating of susceptible and resistant *A. felis* isolates, could be used in segregate analyses.

Although non-synonymous mutations at sites previously associated with azole resistance in *A. fumigatus* were found (M172A, M172V and D255G), these were in AVSC isolates with both high and low azole MICs, and so are unlikely to confer azole resistance, as reported for *A. fumigatus* WT isolates (Howard et al., 2006, Garcia-Effron et al., 2008, Howard et al., 2009, Snelders et al., 2010, Albarrag et al., 2011, Escribano et al., 2011, Mortensen et al., 2011, Buied et al., 2013, Abdolrasouli et al., 2015, Kidd et al., 2015, Lavergne et al., 2015, Mavridou et al., 2015, van Ingen et al., 2015, Meneau et al., 2016, Wiederhold et al., 2016). However, specific combinations of single point mutations may also be important when determining ability to cause azole resistance, as recently demonstrated with *A. fumigatus* isolates harbouring mutations at these positions (M172V and D255E) in combination with either F46Y, G89G, L358L and C454C or F46Y, G89G, N248T, L358L, E427K and C454C were shown to have elevated azole MICs, were pathogenic in *Galleria mellonella* host virulence studies and of a different cluster/lineage to strains of WT *A. fumigatus* (Garcia-Rubio et al., 2018). However, this was not the case for isolates in our study with high azole MIC values that underwent

cyp51A homology modelling.

We identified the azole-resistance associated mutation G138C in two environmental isolates of the *A. felis* clade (DTO 341-E4, DTO 341-E5) from woodland soil in Australia. Interestingly, this mutation was reported in clinical *A. fumigatus* isolates and is thought to be acquired following azole therapy conferring resistance to itraconazole, voriconazole, posaconazole and isavuconazole (Wiederhold et al., 2016), similar to our phenotype results. However, this is not clear, as other single point mutations associated with in-host development have been found in the environment also (e.g. M220 and G54) (Sharma et al., 2015, Bader et al., 2015). These two strains also harboured the T215S mutation, not previously reported for *A. fumigatus*, and not identified in any other isolates in this study. This mutation, in combination with G138C, may also contribute to acquired resistance, however this was not supported by our *cyp51A* homology modelling of AVSC or *A. fumigatus* species. Like our finding, it has been demonstrated via *A. fumigatus cyp51A* homology modelling that while G138C is closely positioned to the protein access channel opening, the mutation can increase stability for azole docking (Liu et al., 2016).

The mutation V101L was found in two *A. aureolus* isolates; however only one sporulated adequately for antifungal susceptibility testing, demonstrating a MIC at the *A. fumigatus* ECV of posaconazole. Mutations at this position in *A. fumigatus* have led to the change V101F, which in association with M220I confers multi-azole resistance (itraconazole and posaconazole resistance with variable voriconazole resistance) (Mortensen et al., 2010). Mutations at M220 were not present in these or any other AVSC isolates. *Cyp51A* homology modelling of these mutations in AVSC and *A. fumigatus* models did not confer azole resistance.

Poor clinical outcomes and high *in vitro* MICs are common amongst patients with aspergillosis due to AVSC member species. We tested AVSC isolate susceptibility to a newer antifungal drug, luliconazole, using the CLSI method. Luliconazole is a cytochrome P450 2C19 inhibitor imidazole antifungal commercially available in a topical 1% cream for treating fungal skin infections. Although there is limited efficacy data for its use in treating aspergillosis, *in vitro* antifungal susceptibility testing on *A. fumigatus* and *A. terreus* isolates and *in vivo* animal models demonstrated a promising treatment alternative to current antifungals, with much lower MIC values and improved survival rates with oral treatments for models of invasive aspergillosis in mice, and intravenous preparation for rat models of pulmonary aspergillosis (Niwano et al., 1999, Abastabar et al., 2016, Zargaran et al., 2017). All but one of our AVSC isolates had MICs in the same range reported for azole susceptible *A. fumigatus* isolates against

luliconazole (0.001 – 0.008 mg/L) (Abastabar et al., 2016). The remaining isolate (*A. udagawae*, soil, USA, pan-azole high MIC phenotype) had a MIC of 1 mg/L, which is higher than the reported range for azole susceptible and resistant *A. fumigatus* isolates (Abastabar et al., 2016). Reference ranges were generally lower than other azoles tested, consistent with the literature for other *Aspergillus* species (Niwano et al., 1999, Zargaran et al., 2017). Although further evaluation is required, this antifungal drug has promising potential as an alternative to current azole therapeutics for *in vivo* therapeutic use on AVSC species.

In this study, high azole MIC values were common in AVSC clinical and environmental isolates. High isavuconazole MICs were most frequent, followed by voriconazole and itraconazole, while high MICs to posaconazole were uncommon. These results are consistent with a recent study of AVSC isolates which found high MICs to voriconazole and itraconazole, with generally low MICs to posaconazole; the study did not test isavuconazole susceptibility (Lyskova et al., 2018). Reference susceptibility testing relies on standardized spore densities for inoculation. This can be difficult in slowly sporulating organisms of the AVSC and 19 isolates failed to sporulate well enough to test. Although *cyp51A* sequences were still determined in these isolates, genotype interpretation is limited without phenotype results.

Other mechanisms of innate and acquired azole resistance should be further investigated as possible causes of AVSC resistance. This could include investigating alterations to other genes related to the development of azole resistance in *A. fumigatus* species, including *cyp51B*, the P88L mutation in the *hapE* gene, *afyap1, aldA, alga* and *AfCox10* genes, multidrug resistant efflux pump genes (*AfuMDR3* and *AfuMDR4*) and the transcriptional regulator *SrbA* (Nascimento et al., 2003, Willger et al., 2008, Camps et al., 2012). Extrolite production could also account for innate resistance profiles in some species and this should be examined. Extrolite products in a limited number of isolates from the complex demonstrated unique properties, including antibacterial and anti-cancer (Lillehoj and Ciegler, 1972, Lillehoj and Milburn, 1973, Mendes et al., 2016, Gonçalves et al., 2016, Frisvad and Larsen, 2016). Examples of AVSC extrolites include viriditoxin, which is produced by all AVSC member species; pyriopyropene A, aspochalasin E and cytochalasins produced by *A. felis*; fumigaclavine C, helvolic acid, methyl-sulochrin, pyripyropene A and E and trypacidin production by *A. udagawae* and *A. viridinutans sensu stricto*; fumigaclavine A and fumiquinazoline F/G in *A. udagawae*; and fumagillin, fumitremorgins A and C, and pyripyropenes O and S (Lillehoj and Ciegler, 1972, Naruse et al., 1993, Xiao et al., 2013, Mendes et al., 2016).

Amplification of *cyp51A* in members of the AVSC identified several non-synonymous mutations within the group compared to the reference sequence for *A. fumigatus*; however, mutations related to environmentally acquired resistance in *A. fumigatus* were not identified. The single point mutation G138C, identified to confer multi and pan azole resistance in *A. fumigatus* clinical isolates associated with therapeutic exposure to azoles, was found in two environmental *A. felis* isolates (woodland soil, Australia). No other mutations commonly associated with azole resistance in *A. fumigatus* were identified in azole resistant AVSC isolates alone. All members of the AVSC (both high and low MICs) had the mutations W6L, V15M, K80R, D255G, C270S, I367L, H403Y, L464I and M172V/A. A total of 13 single point mutations were identified amongst isolates with high azole MICs absent from isolates with low azole MICs; however, none conferred the same resistant phenotype. *Cyp51A* homology modelling of mutations found only in isolates with high azole MICs compared to a low azole MIC isolate model did not significantly change the overall protein structure or directly interfere with azole drug binding to confer resistance. The *cyp51A* gene confers good phylogenetic analysis for the AVSC but is unlikely to enable azole resistance in isolates with high MICs to one or more azole drugs (95 % of AVSC isolates tested). Mechanisms of innate and other causes of acquired azole resistance should be further investigated for the AVSC. Luliconazole showed promise as a therapeutic option by inhibiting the growth of all AVSC isolates on CLSI antifungal susceptibility testing.

Acknowledgements

This research was part funded by an Endeavour Scholarship from the Australian Government for Jessica J. Talbot. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The authors wish to thank the BSc students Nina Adolfse and Pam Heijmans at CWZ for antifungal susceptibility testing.

Tables

Table 1. Comparison of CLSI testing results for clinical (n=17) versus environmental (n=17) AVSC isolates.

Parameter (mg/L)	**ITC**	**POS**	**VRC**	**ISA**	**LUL**
MIC range					
Clinical	0.125->16.00	0.031-0.25	0.25-8.00	0.50-4.00	0.001-0.004
Environmental	0.5->16.00	0.125-0.50	2.00-16.00	2.00-8.00	0.001-1.00
GM MIC					
Clinical	2.76	0.16	2.40	2.40	0.002
Environmental	5.5	0.26	4.69	3.79	0.003
MIC_{50}					
Clinical	1.00	0.25	2.00	2.00	0.002
Environmental	>16.00	0.25	4.00	4.00	0.002
MIC_{90}					
Clinical	>16.00	0.25	4.00	4.00	0.002
Environmental	>16.00	0.50	8.00	8.00	0.004

Note: Three isolates with unknown origin (DTO 019-D8, DTO 316-F9 and DTO 342-I3) were excluded from this table. Abbreviations: ITC, itraconazole; POS, posaconazole; VRC, voriconazole; ISA, isavuconazole; LUL, luliconazole; MIC, minimum inhibitory concentration.

Table 2. *Cyp51A* mutations observed in high azole minimum inhibitory concentration phenotype *Aspergillus viridinutans* species complex isolates (determined by CLSI testing) that were not found in low azole MIC isolates (see Supplementary Table 3 for a list of all mutations found).

Azole resistant phenotype	**Species (isolate number)**	***cyp51A* mutations**
High multi-azole MICs (ITC, ISA, VRC)	*A. felis* clade (DTO 341-E5)	T215S G138C
High multi-azole MICs (ITC, ISA, VRC)	*A. felis* clade (DTO 341-E4)	T215S G138C
High multi-azole MICs (ITC, ISA, VRC)	*A. felis* clade (DTO 131-E6)	S197C Q340R
High multi-azole MICs (ITC, ISA, VRC)	*A. viridinutans* (DTO 050-F1)	A63S L327P V396A
Single azole MIC at the ECV for *A. fumigatus* (POS)	*A. aureolus* (DTO 278-B7)	A103T V101L A234V I360V V428I G505R Q423D F478V

Abbreviations: MICs, minimum inhibitory concentrations; ISA isavuconazole; ITC itraconazole; VRC voriconazole; POS posaconazole; ECV, epidemiological cut-off value.

Table 3. Primers used to target the *cyp51A* gene in AVSC members in this study.

Primer Sets for *cyp51A*	**Primer Pairs used**	**Sequence (5' to 3')**
Aspergillus udagawae		
Forward 1	CYP0-F_uda ¥	GACTTTCATATCTTGCTCAGC
Reverse 1	CYP1-R_uda ¥	AGCCTTGAAAGTTCGGCGAG
Forward 2	CYP2-L*	CATGTGCCACTTATTGAGAAGG
Reverse 2	CYP2-R_uda ¥	CCTTGCGCATGAGCGAGTGA
Forward 3	CYP3-L _uda ¥	TTCCTCCGCTCCAGTACGAG
Reverse 3	CYP3-R_uda ¥	CCTTTGATGTCCTCGATGAAA
Forward 4	CYP3-L _uda ¥	TTCCTCCGCTCCAGTACGAG
Reverse 4	CYP4-R_uda ¥	GATCGCACCGTGTCCTTTG
Degenerate Primers		
Forward 5	CYP0-F_deg±	GRCKTTCAWATSTTGCTCAGC
Reverse 5	CYP1-R_deg±	AGCCTTGAAARTTCGGYGAR
Forward 6	CYP2-L_deg±	CATGTGCCACTYATYGAGAAGG
Reverse 6	CYP2-R_deg±	CCTTGCGCATGAKMGAGTGA
Forward 7	CYP0-F_deg±	GRCKTTCAWATSTTGCTCAGC
Reverse 7	CYP2-R_deg±	CCTTGCGCATGAKMGAGTGA
Forward 8	CYP3-L*	TTCCTCCGCTCCAGTACAAG
Reverse 8	CYP4-R_deg±	GATCRCACCRWRTCCTTTG

¥ primers identified from contig:Aud0040 of *A. udagawae* (IFM 46973) (Kusuya *et al.*, 2015) corresponding with primers developed by Chen *et al.*, 2005; ***** primers used by Chen *et al.*, 2005; ± degenerate primers developed for this study.

Table 4. Docking scores of the best conformers of posaconazole, itraconazole and voriconazole, and corresponding MMGBSA study values. Models F to J are *Aspergillus viridinutans* species complex member based models and models B, K and L are *A. fumigatus based* models.

Model (species)	**Ligand**	**Docking score (kcal/mol)**	**Ligand strain energy (kcal/mol)**	**Receptor strain energy (kcal/mol)**	**ΔG(binding) (kcal/mol)**
F (*A. felis* clade)	posaconazole #2	-9.549	20.174	43.264	-145.031
	itraconazole #2	-10.348	10.148	61.584	-148.403
	voriconazole	-7.284	2.101	18.284	-56.913
G (*A. viridinutans)*	posaconazole #2	-9.457	17.582	20.2	-148.061
	itraconazole #1	-7.972	11.809	48.485	-117.783
	voriconazole	-7.13	2.618	23.192	-51.94
H (*A. aureolus)*	posaconazole #2	-10.21	11.18	62.408	-117.291
	itraconazole #4	-8.732	8.419	29.881	-134.629
	voriconazole	-7.881	10.41	16.574	-64.09
I (*A. felis* clade mutations T215S/G138C)	posaconazole #2	-11.541	13.355	34.947	-140.137
	itraconazole #1	-11.031	10.476	36.487	-122.160
	voriconazole	-7.220	6.929	20.331	-50.546
J (*A. felis* clade mutations S197C/Q340R)	posaconazole #2	-10.275	16.779	36.947	-141.408
	itraconazole #3	-10.318	14.646	36.402	-112.306
	voriconazole	-7.461	6.916	24.457	-50.255
B (*A. fumigatus*)	posaconazole #2	-10.385	11.936	34.031	-133.210
	itraconazole #4	-9.543	11.503	10.276	-128.252
	voriconazole	-6.991	2.048	-0.416	-49.793
K (*A. fumigatus* model of model I mutations)	posaconazole #2	-9.859	12.880	30.784	-145.495
	itraconazole #4	-9.002	11.412	52.439	-131.727
	voriconazole	-7.570	11.101	-0.489	-72.311
L (*A. fumigatus* model of V101L/A103T/A234V /I360V/V428I/G505R `/Q423D/F478V)	posaconazole #1	-10.530	12.928	29.622	-122.594
	itraconazole #2	-10.764	10.357	49.188	-148.968
	voriconazole	-7.098	2.482	31.636	-49.211

Figures

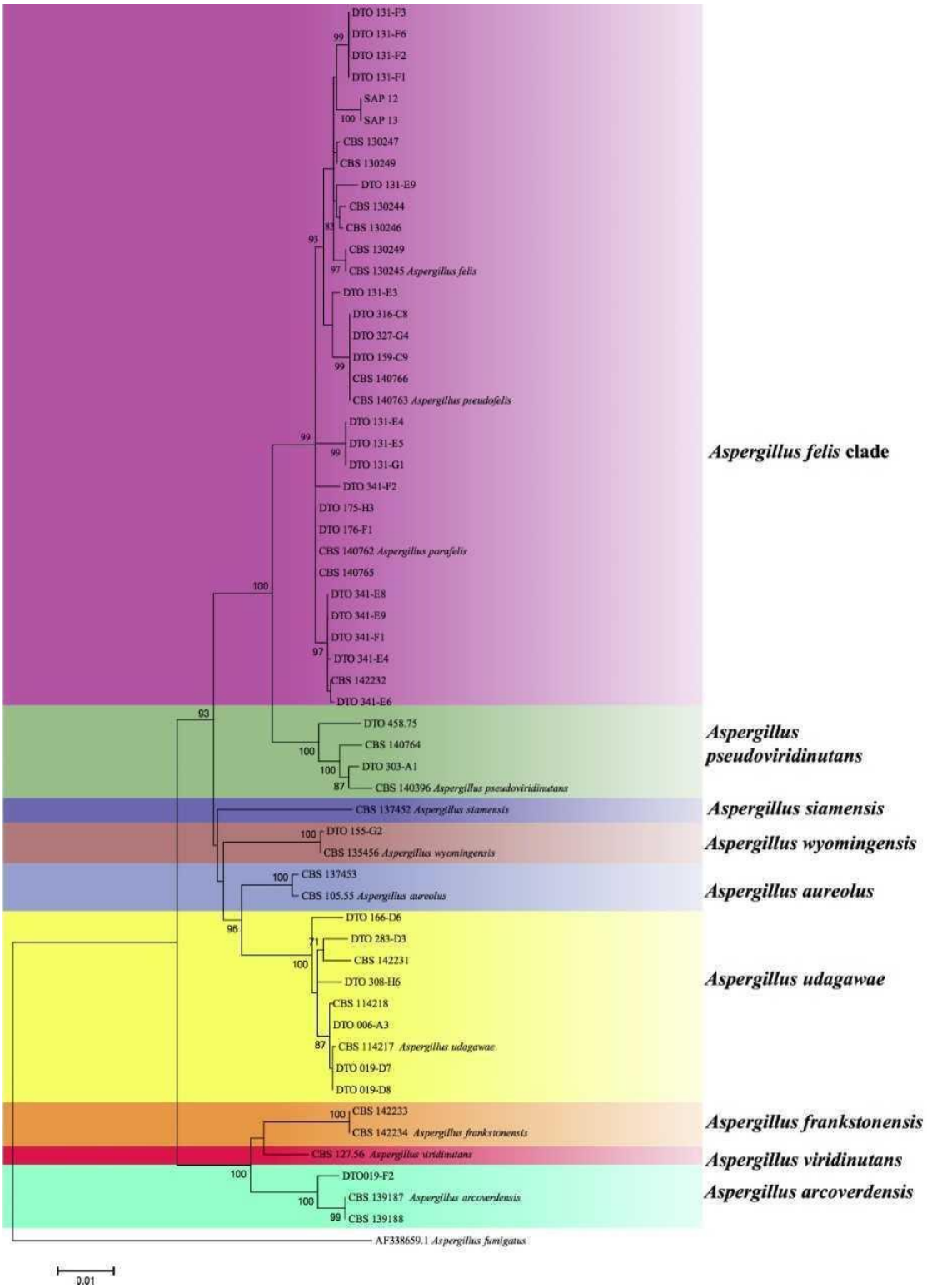


Figure 1. Phylogenetic tree of *cyp51A* sequences

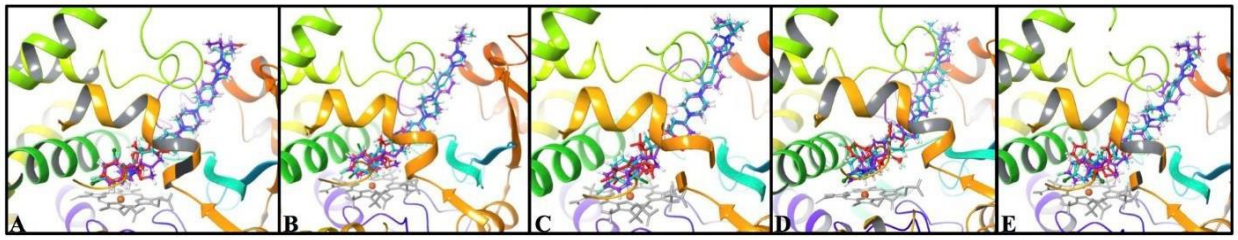
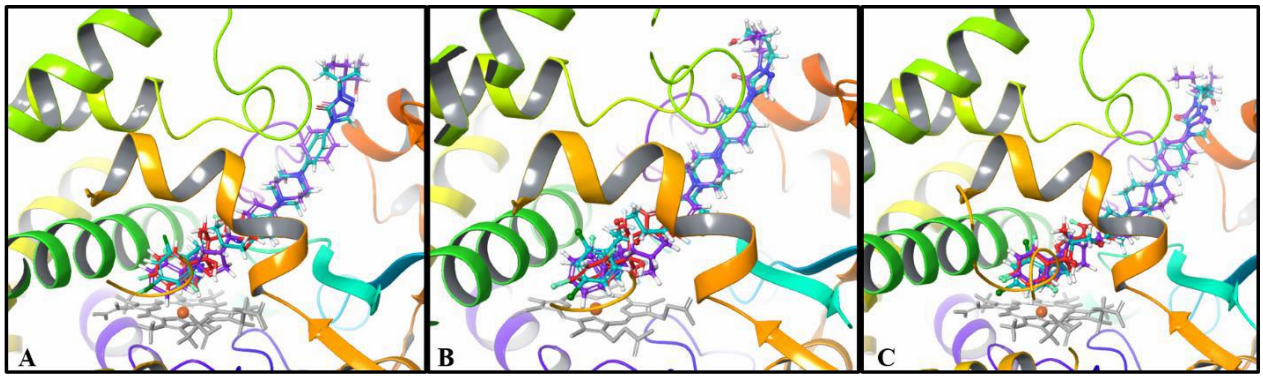


Figure 2. Voriconazole (red), itraconazole (blue) and posaconazole (purple) binding to the haem group (grey) of *cyp51A* homology models F (*A. felis* clade [A]), G (*A. viridinutans* [B]), H (*A. aureolus* [C]), I and J (*A. felis* clade [D and E]).

Supplementary data



Supplementary Figure 1. Voriconazole (red), itraconazole (blue) and posaconazole (purple) binding to the haem group (grey) of *cyp51A* homology models B (*A. fumigatus* wild-type [A]), K (*A. fumigatus* [B], and L (*A. fumigatus* [C]).

Supplementary Tables

Supplementary Table 1. All isolates used in this study, their associated *cyp51A* GenBank accession number and antifungal susceptibility testing method used.

Strain number	**Species**	**Source**	**Location**	**GenBank accession number**	**Antifungal susceptibility testing**
DTO 019-F2	*A. arcoverdensis*	soil	Australia	MF178320	-
DTO 316-F7 (= CBS 139187 (T))	*A. arcoverdensis*	semi-desert soil	Brazil	MF178321	-
DTO 316-F9 (= CBS 139188)	*A. arcoverdensis*	unknown	Brazil	MF178322	CLSI
DTO 278-B7 (= CBS 137453)	*A. aureolus*	soil	Brazil	MF178323	CLSI
DTO 331-G6 (= CBS 105.55 (T) = DTO 052-C8 = NRRL 2244)	*A. aureolus*	soil	Ghana	MF178324	-
CBS 143249	*A. felis*-clade	human	Australia	MF178299	CLSI
DTO 131-E3	*A. felis*-clade	cat, RBM	Australia	MF178286	-
DTO 131-E4	*A. felis*-clade	cat, RBM	Australia	MF178301	CLSI
DTO 131-E5	*A. felis*-clade	cat, RBM	Australia	MF178302	CLSI
DTO 131-E6 (= CBS 130244)	*A. felis*-clade	cat, RBM	Australia	MF178292	CLSI
DTO 131-E9	*A. felis*-clade	cat, RBM	Australia	MF178287	-
DTO 131-F1	*A. felis*-clade	cat, RBM	Australia	MF178288	CLSI
DTO 131-F2	*A. felis*-clade	cat, RBM	Australia	MF178289	-
DTO 131-F3	*A. felis*-clade	cat, RBM	Australia	MF178290	CLSI
DTO 131-F4 (= CBS 130245 (T))	*A. felis*-clade	cat, RBM	Australia	MF178296	-
DTO 131-F6	*A. felis*-clade	cat, RBM	Australia	MF178291	CLSI
DTO 131-F9 (= CBS 130246)	*A. felis*-clade	cat, SNC	Australia	MF178297	-
DTO 131-G1	*A. felis*-clade	cat, RBM	Australia	MF178303	CLSI
DTO 131-G2 (= CBS 130247)	*A. felis*-clade	cat, RBM	Australia	MF178293	-
DTO 131-G3 (= CBS 130248)	*A. felis*-clade	cat, RBM	Australia	MF178294	CLSI
DTO 155-G3 (= CBS 130249)	*A. felis*-clade	dog, VH	Australia	MF178295	CLSI

DTO 159-C9 (= CBS 130250)	*A. felis*-clade	cat, RBM	United Kingdom	MF178317	-
DTO 175-H3	*A. felis*-clade	surface water	Portugal	MF178304	CLSI
DTO 176-F1	*A. felis*-clade	air	Germany	MF178305	CLSI
DTO 316-C8	*A. felis*-clade	CBS culture contaminant	The Netherlands	MF178316	CLSI
DTO 327-G4	*A. felis*-clade	human patient	The Netherlands	MF178315	CLSI
DTO 341-E4	*A. felis*-clade	woodland soil	Frankston, Australia	MF178306	CLSI
DTO 341-E5 (= CBS 142232)	*A. felis*-clade	woodland soil	Frankston, Australia	MF178307	CLSI
DTO 341-E6	*A. felis*-clade	woodland soil	Frankston, Australia	MF178308	-
DTO 341-E8	*A. felis*-clade	woodland soil	Frankston, Australia	MF178309	CLSI
DTO 341-E9	*A. felis*-clade	woodland soil	Frankston, Australia	MF178310	CLSI
DTO 341-F1	*A. felis*-clade	woodland soil	Frankston, Australia	MF178311	-
DTO 341-F2	*A. felis*-clade	woodland soil	Frankston, Australia	MF178312	CLSI
DTO 342-I4 (= NRRL 62900 = CM-3147 = DTO 342-I4 = CBS 140762 (T))	*A. felis*-clade	human, OPE	Spain	MF178313	CLSI
DTO 342-I5 (= NRRL 62901 = CM-5623 = DTO 342-I5 = CBS 140765)	*A. felis*-clade	human, lungs	Portugal	MF178314	CLSI
DTO 342-I6 (= NRRL 62902 = CM-4518 = DTO 342-I6 = CBS 140766)	*A. felis*-clade	human, nail	Spain	MF178318	CLSI
DTO 342-I7 (= NRRL 62903 = CM-6087 = DTO 342-I7 = CBS 140763 (T))	*A. felis*-clade	human, sputum	Spain	MF178319	CLSI
DTO 341-E7 (= CBS 142233 = IBT 34172 (T))	*A. frankstonensis*	woodland soil	Frankston, Australia	MF178271	-
DTO 341-F3 (= CBS 142234 = IBT 34204)	*A. frankstonensis*	woodland soil	Frankston, Australia	MF178272	CLSI
DTO 153-A1 (= CBS 458.75)	*A. pseudoviridinutans*	soil	India	MF178276	-
DTO 303-A1	*A. pseudoviridinutans*	*Pinus caribea* (pine tree)	Sri Lanka	MF178273	CLSI
DTO 304-I5 (= NRRL 62904 = NIHAV1 = DTO 304-I5 = CBS 140396 (T))	*A. pseudoviridinutans*	human, lung	USA	MF178275	-
DTO 342-I3 (= NRRL 6106 = DTO 342-I3 = CBS 140764)	*A. pseudoviridinutans*	unknown	unknown	MF178274	CLSI
DTO 278-B6 (= CBS 137452 (T))	*A. siamensis*	soil	Thailand	MF178325	-
DTO 006-A3	*A. udagawae*	soil	USA	MF178277	CLSI
DTO 019-D7	*A. udagawae*	unknown	unknown	MF178280	-
DTO 019-D8	*A. udagawae*	unknown	unknown	MF178281	CLSI

DTO 157-D7 (= CBS 114217 (T))	*A. udagawae*	soil	Brazil	MF178278	CLSI
DTO 157-D8 (= CBS 114218)	*A. udagawae*	soil	Brazil	MF178279	CLSI
DTO 166-D6	*A. udagawae*	cat	Australia	MF178282	CLSI
DTO 283-D3	*A. udagawae*	soil	Thailand	MF178283	-
DTO 308-H6	*A. udagawae*	soil	Turkey	MF178284	CLSI
DTO 341-E3 (= CBS 142231)	*A. udagawae*	cat, RBM	Kealba, Australia	MF178285	CLSI
DTO 050-F1 (= CBS 127.56 (T) = NRRL 4365)	*A. viridinutans*	rabbit dung	Australia	MF178270	CLSI
DTO 155-G2	*A. wyomingensis*	cat, RBM	Australia	MF178298	-
DTO 332-B1 (= CBS 135456 (T))	*A. wyomingensis*	coal mine reclamation site soil	Glenrock, USA	MF178326	CLSI

Abbreviations: (T), type strain; CLSI, Clinical Laboratory Standards Institute; - = isolate did not sporulate for testing.

Supplementary Table 2. CLSI antifungal susceptibility testing results for 37 *Aspergillus viridinutans* species complex isolates included in this study.

Drug	Species	MIC/MEC (μg/mL) distribution among tested isolates															
		0.001	**0.002**	**0.004**	**0.008**	**0.015**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>16**	**GM**
ITZ*	*A. arcoverdensis*									1								0.25
	A. aureolus									1								0.25
	A. felis								1	2	4	1				1	14	4.38
	A. frankstonensis																1	>16
	A. pseudoviridinutans									1							1	4
	A. udagawae									1	5						1	1.26
	A. viridinutans																1	>16
	A. wyomingensis																1	>16
VCZ*	*A. arcoverdensis*										1							0.5
	A. aureolus										1							0.5
	A. felis									1	2		4	13	3			2.87
	A. frankstonensis														1			8
	A. pseudoviridinutans										1			1				1.41
	A. udagawae												5	1		1		2.97
	A. viridinutans													1				4
	A. wyomingensis													1				4
POS*	*A. arcoverdensis*							1										0.06
	A. aureolus										1							0.5
	A. felis						2		4	15	2							0.2
	A. frankstonensis										1							0.5
	A. pseudoviridinutans							1		1								0.25
	A. udagawae								3	3							1	0.34

	A. viridinutans									1								0.25
	A. wyomingensis									1								0.25
ISA*	*A. arcoverdensis*											1						1
	A. aureolus								1									0.13
	A. felis										2	1	6	12	2			2.79
	A. frankstonensis														1			8
	A. pseudoviridinutans										1			1				1.41
	A. udagawae											1	5		1			2.21
	A. viridinutans													1				4
	A. wyomingensis														1			8
AMB*	*A. arcoverdensis*								1									0.13
	A. aureolus											1						1
	A. felis									2	1	14	6					1.03
	A. frankstonensis												1					2
	A. pseudoviridinutans								1			1						0.35
	A. udagawae										1	3	3					1.22
	A. viridinutans										1							0.5
	A. wyomingensis											1						1
MIF†	*A. arcoverdensis*								1									0.13
	A. aureolus											1						1
	A. felis						4	5	3	2	1	1			1		6	0.44
	A. frankstonensis							1										0.06
	A. pseudoviridinutans							2										0.06
	A. udagawae							3	1	1	1		1					0.19
	A. viridinutans								1									0.125
	A. wyomingensis																1	>16
LUL*	*A. arcoverdensis*	1																0
	A. aureolus			1														0

	A. felis	7	15	1														0
	A. frankstonensis		1															0
	A. pseudoviridinutans	1	1															0
	A. udagawae	3	3									1						0
	A. viridinutans		1															0
	A. wyomingensis			1														0

Abbreviations: ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; ISA, isavuconazole; AMB, amphotericin B; MIF, micafungin; LUL, luliconazole. *minimum inhibitory concentration values calculated; † minimum effective concentration calculated.

Supplementary Table 3. All AVSC *cyp51A* mutations found in this study compared to *A. fumigatus* wild-type *cyp51A*.

Mutation present	**Isolate ID/ Species**
P3S, V13G	6 *A. felis*-clade isolates (DTO 341-E4, DTO 341-E5, DTO 341-E6, DTO 341-E8, DTO 341-E9, DTO 341-F1)
L5V	1 *A. wyomingensis* isolate (DTO 155-G2)
M11L, S493T	All 4 *A. pseudoviridinutans* and 32 *A. felis*-clade isolates
A12V, L31H, T35A, Y137F, I151V, P376S, C409R, E488K, N512V	All 1 *A. siamensis* isolates (DTO 278-B6)
A18V	All 1 *A. viridinutans*, 2 *A. frankstonensis*, 9 *A. udagawae*, 3 *A. arcoverdensis*, 2 *A. aureolus*, 1 *A. siamensis* and 2 *A. wyomingensis* isolates
I19T, V44F, A177V	All 2 *A. frankstonensis* isolates
I19V, L316P	6 *A. felis*-clade isolates (DTO 131-E3, DTO 327-G4, DTO 316-C8, DTO 159-C9, DTO 342-I6, DTO 342-I7)
F28L, D253G, M499V	1 *A. pseudoviridinutans* isolate (DTO 153-A1)
F29Y, P109A, N512E	All 3 *A. arcoverdensis* isolates
T35I, S117T, A330T	1 *A. udagawae* isolate (DTO 283-D3)
M39T, V480M	3 *A. felis*-clade isolates (DTO 131-E4, DTO 131-E5, DTO 131-G1)
V44I, E317D, M383L, Q423H	All 9 *A.udagawae* isolates
T50A	All 4 *A. pseudoviridinutans* isolates
K59R	All 1 *A. viridinutans* and 2 *A. frankstonensis* isolates
A63S, L327P, V396A	All 1 *A. viridinutans* isolates
I71V	1 *A. arcoverdensis* isolate (DTO 019-F2)
V101L, A103T, A234V, I360V, V428I, G505R, Q423D, F478V	All 2 *A. aureolus* isolates
T215S, G138C	2 *A. felis*-clade isolates (DTO 341-E4, DTO 341-E5)
D161N	All AVSC isolates (except 2 *A. udagawae* isolates [DTO 283-D3 and DTO 341-E3])
R171H	All 2 *A. frankstonensis* and 3 *A. arcoverdensis* isolates
R171K	6 *A. felis*-clade isolates (CBS 143249, DTO 327-G4, DTO 316-C8, DTO 159-C9, DTO 342-I6, DTO 342-I7)
R171Q	All 1 *A. siamensis*, 2 *A. wyomingensis* and 31*A. felis*-clade isolates (all *A. felis*-clade except CBS 143249)

M172A	1 *A. udagawae* isolate (DTO 308-H6)
A189V	1 *A. pseudoviridinutans* isolate (DTO 342-I3)
S197C, Q340R	1 *A. felis*-clade isolate (DTO 131-E6)
A234T, S241A	3 *A. pseudoviridinutans* isolates (DTO 303-A1, DTO 342-I3, DTO 304-I5)
K256N	All 2 *A. aureolus* and 1 *A. arcoverdensis* (DTO 019-F2) isolates
K256R, L390P, H474Y	1 *A. pseudoviridinutans* isolate (DTO 304-I5)
D257E, K346R	All 1 *A. viridinutans*, 2 *A. frankstonensis* and 3 *A. arcoverdensis* isolates
N274S	19 *A. felis*-clade isolates (DTO 327-G4, DTO 316-C8, DTO 159-C9, DTO 342-I6, DTO 342-I7, CBS 143249, DTO 131-E3, DTO 131-E9, DTO 131-G3, DTO 131-F4, DTO 131-F1, DTO 131-F2, DTO 131-F3, DTO 131-G2, DTO 155-G3, DTO 131-F9, DTO 131-F6, DTO 131-E6, DTO 155-G2)
K281H	All 4 *A. pseudoviridinutans* isolates
K281N, K314D	All 32 *A. felis*-clade isolates
S302G	All 4 *A. pseudoviridinutans*, 9 *A. udagawae*, 1 *A. siamensis*, 2 *A. wyomingensis*, and 31 *A. felis*-clade isolates (all *A. felis*-clade isolates except CBS 143249)
K314E	All 1 *A. viridinutans*, 2 *A. frankstonensis*, 4 *A. pseudoviridinutans*, 9 *A. udagawae*, 3 *A. arcoverdensis*, 2 *A. aureolus*, 1 *A. siamensis* and 2 *A. wyomingensis* isolates
Q321R, L344I	1 *A. felis*-clade isolate (DTO 341-F2)
N326S	10 *A. felis*-clade isolates (DTO 131-E3, DTO 131-E9, DTO 131-G3, DTO 131-F4, CBS 143249, DTO 327-G4, DTO 316-C8, DTO 159-C9, DTO 342-I6, DTO 342-I7)
A330I	All AVSC isolates (except 1 *A. pseudoviridinutans* [DTO 303-A1] and 1 *A. udagawae* [DTO 283-D3])
K342E	8 *A. udagawae* isolates (DTO 006-A3, DTO 019-D7, DTO 019-D8, DTO 157-D8, DTO 166-D6, DTO 283-D3, DTO 308-H6, DTO 341-E3)
I354V, M383V	All AVSC isolates (except all 9 *A. udagawae* isolates)
I360L, V462L	All AVSC isolates (except all 2 *A. aureolus* isolates)
S373N	All 4 *A. pseudoviridinutans*, 2 *A. wyomingensis* and 31 *A. felis*-clade isolates (all *A. felis*-clade isolates except DTO 131-F6)
S373R	2 *A. arcoverdensis* isolates (DTO 316-F7 and DTO 316-F9)
L375F, C409Y, A419D	1 *A. udagawae* isolate (DTO 166-D6)
I384V	1 *A. udagawae* isolate DTO 341-E3, all 3 *A. arcoverdensis* isolates
P386S, M499K	1 *A. pseudoviridinutans* isolate (DTO 303-A1)
N406S	All 4 *A. pseudoviridinutans*, 2 *A. wyomingensis* and 32 *A. felis*-clade isolates

T420A	All 1 *A. viridinutans*, 4 *A. pseudoviridinutans*, 2 *A. aureolus*, 1 *A. siamensis*, 2 *A. wyomingensis*, and 32 *A. felis*-clade isolates, and 3 *A. udagawae* isolates (DTO 166-D6, DTO 308-H6, DTO 341-E3)
T420S	5 *A. udagawae* isolates (DTO 157-D7, DTO 157-D8, DTO 019-D7, DTO 019-D8, DTO 283-D3)
T420V	All 2 *A. frankstonensis* and 3 *A. arcoverdensis* isolates
N425S	31 *A. felis*-clade isolates (all *A. felis*-clade isolates except DTO 341-E5)
V428F	17 *A. felis*-clade isolates (DTO 131-E3, DTO 131-E6, DTO 131-E9, DTO 131-F1, DTO 131-F2, DTO 131-F4, DTO 131-F6, DTO 131-F9, DTO 131-G2 DTO 131-G3, DTO 155-G3, DTO 159-C9, DTO 316-C9, DTO 327-G4, DTO 342-I6, DTO 342-I7, CBS 143249)
V428L	1 *A. pseudoviridinutans* isolate (DTO 153-A1), 3 *A. udagawae* isolates (DTO 157-D7, DTO 019-D7, DTO 019-D8)
I471F	1 *A. aureolus* isolate (DTO 331-G6)
I471V	All AVC isolates (except 2 *A. arcoverdensis* [DTO 316-F7 and DTO 316-F9] and one *A. aureolus* [DTO 331-G6] isolates)
V480A	All 2 *A. wyomingensis* isolates
G482E	All 9 *A. udagawae* and 2 *A. wyomingensis* isolates
M499I	5 *A. felis*-clade isolates (DTO 327-G4, DTO 316-C8, DTO 159-C9, DTO 342-I6, DTO 342-I7)
N512D	All AVSC isolates (except all 3 *A. arcoverdensis* and 1 *A. siamensis* isolates)
T513V	All 9 *A. udagawae*, 2 *A. aureolus* and 2 *A. wyomingensis* isolates
W6L, V15M, K80R, M172V[¥], D255G, C270S, I367L, H403Y, L464I	All 56 AVSC isolates

[¥]55/56 AVSC isolates tested had this mutation, and the remaining isolate (DTO 308-H6 [*A. udagawae*]) had a mutation at this position of M172A.

Supplementary Table 4. Verify3D results of the five *A. fumigatus* homology models (Model A-E) and three AVSC homology models (Model F-H).

Model	Species (isolate number)	% of residues that have scored a 3D-1D score >=0.2
A	*A. fumigatus* (AF338659)	83.11
B	*A. fumigatus* (AF338659)	84.66
C	*A. fumigatus* (AF338659)	84.47
D	*A. fumigatus* (AF338659)	84.08
E	*A. fumigatus* (AF338659)	84.65
F	*A. felis* (DTO 131-F6)	85.41
G	*A. viridinutans* (DTO 050-F1)	83.50
H	*A. aureolus* (DTO 278-B7)	82.91

7.4 Conclusions

This investigation showed that while most AVSC members have high MICs of one or more triazole antifungal drugs and contain many single point mutations in the *cyp51A* gene compared to the *A. fumigatus* wild genotype, these mutations are unlikely to confer the observed azole resistance. Very few known azole resistance mechanisms were identified, and comparison of azole susceptible and resistant isolates showed few mutations were only associated with isolates considered resistant and these could not be confirmed to confer azole resistance on *cyp51A* homology modelling. On CLSI testing, 84% of isolates demonstrated high MIC values against azole drugs that are considered resistant phenotype in *A. fumigatus* species, and resistance was observed in clinical and environmental isolates, with no statistically significant differences between these groups. Thus, this research highlights the need to further investigate inherent defence mechanisms in this species complex and novel antifungal drug alternatives to available therapies.

During my PhD I also performed secondary metabolite extraction and HPLC analysis on these AVSC isolates under the supervision and expertise of Prof Jens Frisvad at the Technical University of Denmark. We are still investigating the information collected and will continue to examine post this thesis.

This research also highlighted differences in the susceptibility testing results between CLSI and Sensititre™ antifungal susceptibility testing methods, like the findings of Lyskova et al. (2018) who compared EUCAST and Sensititre™ testing methods. In their study Sensititre™ could not detect elevated itraconazole MICs (Lyskova et al., 2018), and this was also true for most isolates in our study. In Australia Sensititre™ commercial testing is used by the Australian National Reference Laboratory in Medical Mycology following the preparation standards set for moulds in the CLSI M38 method, as EUCAST and CLSI methods are not routinely available in the region (Ellis, 2017, Kidd et al., 2017, Clinical Laboartory Standards Institute (CLSI), 2017). While Sensititre™ has been demonstrated to provide comparable results with the CLSI method for *A. fumigatus*, the results of this research show they are not comparable for AVSC species, and it is recommended Sensititre™ is not used for antifungal susceptibility testing for these species where CLSI or EUCAST testing can be performed.

7.5 References

ABASTABAR, M., RAHIMI, N., MEIS, J. F., ASLANI, N., KHODAVAISY, S., NABILI, M., REZAEI-MATEHKOLAEI, A., MAKIMURA, K. & BADALI, H. 2016. Potent activities of novel imidazoles lanoconazole and luliconazole against a collection of azole-resistant and -susceptible *Aspergillus fumigatus* strains. *Antimicrobial Agents and Chemotherapy,* 60**,** 6916-6919.

ABDOLRASOULI, A., RHODES, J., BEALE, M. A., HAGEN, F., ROGERS, T. R., CHOWDHARY, A., MEIS, J. F., ARMSTRONG-JAMES, D. & FISHER, M. C. 2015. Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. *mBio,* 6**,** e00536.

ALBARRAG, A. M., ANDERSON, M. J., HOWARD, S. J., ROBSON, G. D., WARN, P. A., SANGLARD, D. & DENNING, D. W. 2011. Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in cyp51A, upregulation of *cyp51A*, and integration and activation of transposon Atf1 in the *cyp51A* promoter. *Antimicrobial Agents and Chemotherapy,* 55**,** 5113-21.

ALCAZAR-FUOLI, L., MELLADO, E., ALASTRUEY-IZQUIERDO, A., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2008. *Aspergillus* section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. *Antimicrobial Agents and Chemotherapy,* 52**,** 1244-1251.

ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., BLANCO, J. L., GARCIA, M. E., KWON, M., MUÑOZ, P., CUENCA-ESTRELLA, M., BOUZA, E. & PELÁEZ, T. 2014. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. *Journal of Medical Microbiology,* 63**,** 617-619.

ARENDRUP, M. C., MELETIADIS, J., MOUTON, J. W., LAGROU, K., HAMAL, P., GUINEA, J. & EUCAST-AFST 2017. EUCAST antifungal MIC method for conidia forming moulds. *EUCAST Definitive Document E.DEF.9.3.1.* Copenhagen, Denmark: EUCAST.

BADER, O., TUNNERMANN, J., DUDAKOVA, A., TANGWATTANACHULEEPORN, M., WEIG, M. & GROSS, U. 2015. Environmental isolates of azole-resistant *Aspergillus fumigatus* in Germany. *Antimicrobial Agents and Chemotherapy,* 59**,** 4356-9.

BALAJEE, S. A., NICKLE, D., VARGA, J. & MARR, K. A. 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryotic Cell,* 5**,** 1705-1712.

BARRS, V. R., VAN DOORN, T. M., HOUBRAKEN, J., KIDD, S. E., MARTIN, P., PINHEIRO, M. D., RICHARDSON, M., VARGA, J. & SAMSON, R. A. 2013. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One,* 8**,** e64871.

BOWIE, J. U., LUTHY, R. & EISENBERG, D. 1991. A method to identify protein sequences that fold into a known three-dimensional structure. *Science* 253**,** 164-170.

BUIED, A., MOORE, C. B., DENNING, D. W. & BOWYER, P. 2013. High-level expression of cyp51B in azole-resistant clinical *Aspergillus fumigatus* isolates. *Journal of Antimicrobial Chemotherapy,* 68**,** 512-4.

CAMPS, S. M., DUTILH, B. E., ARENDRUP, M. C., RIJS, A. J. M. M., SNELDERS, E., HUYNEN, M. A., VERWEIJ, P. E. & MELCHERS, W. J. G. 2012. Discovery of a *hapE* mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. *PLoS ONE,* 7**,** e50034.

CHEN, J., LI, H., LI, R., BU, D. & WAN, Z. 2005. Mutations in the cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *Journal of Antimicrobial Chemotherapy,* 55**,** 31-37.

CHOWDHARY, A., KATHURIA, S., XU, J. & MEIS, J. F. 2013. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health *PLoS Pathogens,* 9**,** e1003633.

CHOWDHARY, A. & MEIS, J. F. 2018. Emergence of azole resistant *Aspergillus fumigatus* and One Health: time to implement environmental stewardship. *Environ Microbiol,* 20**,** 1299-1301.

CHOWDHARY, A., SHARMA, C. & MEIS, J. F. 2017. Azole-resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. *The Journal of Infectious Diseases,* 216**,** S436-S444.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) 2018. M59 Epidemiology cutoff values of antifungal susceptibility testing Clinical and Laboratory Standards Institute: 2nd ed. CLSI supplement M59, Wayne, PA.

CLINICAL LABOARTORY STANDARDS INSTITUTE (CLSI) 2017. M38, 3rd edition *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi.* Clinical and Laboratory Standards Institute: CLSI, Wayne, PA.

COELHO, D., SILVA, S., VALE-SILVA, L., GOMES, H., PINTO, E., SARMENTO, A. & PINHEIRO, M. D. 2011. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. *Medical Mycology,* 49**,** 755-759.

DENNING, D. W., VENKATESWARLU, K., OAKLEY, K. L., ANDERSON, M. J., MANNING, N. J., STEVENS, D. A., WARNOCK, D. W. & KELLY, S. L. 1997. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy,* 41**,** 1364-1368.

ELLIS, D. 2017. *Mycology online* [Online]. Adelaide, SA: The University of Adelaide. Available: https://mycology.adelaide.edu.au/laboratory/ 2017].

ESCRIBANO, P., RECIO, S., PELAEZ, T., BOUZA, E. & GUINEA, J. 2011. *Aspergillus fumigatus* strains with mutations in the *cyp51A* gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. *Antimicrobial Agents and Chemotherapy,* 55**,** 2460-2.

ESPINELL-INGROFF, A., DIEKEMA, D. J., FOTHERGILL, A., JOHNSON, E., PELAEZ, T., PFALLER, M. A., RINALDI, M. G., CANTON, E. & TURNIDGE, J. 2010. Wild-type

MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Journal of Clinical Microbiology,* 48**,** 3251-3257.

FRIESNER, R. A., BANKS, J. L., MURPHY, R. B., HALGREN, T. A., KLICIC, J. J., MAINZ, D. T., REPASKY, M. P., KNOLL, E. H., SHAW, D. E., SHELLEY, M., PERRY, J. K., FRANCIS, P. & SHENKIN, P. S. 2004. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy *Journal of Medicinal Chemistry,* 47**,** 1739-1749.

FRIESNER, R. A., MURPHY, R. B., REPASKY, M. P., FRYE, L. L., GREENWOOD, J. R., HALGREN, T. A., SANSCHAGRIN, P. C. & MAINZ, D. T. 2006. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *Journal of Medicinal Chemistry,* 49**,** 6177-6196.

FRISVAD, J. C. & LARSEN, T. O. 2016. Extrolites of *Aspergillus fumigatus* and other pathogenic species in *Aspergillus* section *Fumigati*. *Frontiers in Microbiology,* 6**,** 1-14.

GARCIA-EFFRON, G., DILGER, A., ALCAZAR-FUOLI, L., PARK, S., MELLADO, E. & PERLIN, D. S. 2008. Rapid detection of triazole antifungal resistance in *Aspergillus fumigatus*. *Journal of Clinical Microbiology,* 46**,** 1200-6.

GARCIA-RUBIO, R., ALCAZAR-FUOLI, L., MONTEIRO, M. C., MONZON, S., CUESTA, I., PELAEZ, T. & MELLADO, E. 2018. *Aspergillus fumigatus cyp51A* polymorphisms, an insight on their significance. *Antimicrobial Agents and Chemotherapy*.

GONÇALVES, V. N., CANTRELL, C. L., WEDGE, D. E., FERREIRA, M. C., SOARES, M. A., JACOB, M. R., OLIVEIRA, F. S., GALANTE, D., RODRIGUES, F., ALVES, T. M., ZANI, C. L., JUNIOR, P. A., MURTA, S., ROMANHA, A. J., BARBOSA, E. C., KROON, E. G., OLIVEIRA, J. G., GOMEZ-SILVA, B., GALETOVIC, A., ROSA, C. A. & ROSA, L. H. 2016. Fungi associated with rocks of the Atacama Desert: taxonomy, distribution, diversity, ecology and bioprospection for bioactive compounds. *Environmental Microbiology,* 18**,** 232-245.

GYOTOKU, H., IZUMIKAWA, K., IKEDA, H., TAKAZONO, T., MORINAGA, Y., NAKAMURA, S., IMAMURA, Y., NISHINO, T., MIYAZAKI, T., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., YASUOKA, A., YAGUCHI, T., OHNO, H., MIYZAKI, Y., KAMEI, K., KANDA, T. & KOHNO, S. 2012. A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. *Med Mycol.*, 50**,** 631-636.

HALGREN, T. A., MURPHY, R. B., FRIESNER, R. A., BEARD, H. S., FRYE, L. L., POLLARD, W. T. & BANKS, J. L. 2004. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening *Journal of Medicinal Chemistry*, 47**,** 1750-1759.

HARGROVE, T. Y., WAWRZAK, Z., LAMB, D. C., GUENGERICH, F. P. & LEPESHEVA, G. I. 2015. Structure-functional characterization of cytochrome P450 sterol 14α-demethylase (*CYP51B*) from *Aspergillus fumigatus* and molecular basis for the development of antifungal drugs. *The Journal of Biological Chemistry* 290**,** 23916-23934.

HOWARD, S. J. & ARENDRUP, M. C. 2011. Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection. *Medical Mycology*, 49**,** S90-S95.

HOWARD, S. J., CERAR, D., ANDERSON, M. J., ALBARRAG, A., FISHER, M. C., PASQUALOTTO, A. C., LAVERDIERE, M., ARENDRUP, M. C., PERLIN, D. S. & DENNING, D. W. 2009. Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerging Infectious Diseases,* 15**,** 1068-76.

HOWARD, S. J., WEBSTER, I., MOORE, C. B., GARDINER, R. E., PARK, S., PERLIN, D. S. & DENNING, D. W. 2006. Multi-azole resistance in *Aspergillus fumigatus*. *International Journal of Antimicrobial Agents*, 28**,** 450-3.

HUBKA, V., BARRS, V., DUDOVA, Z., SKLENAR, F., KUBATOVA, A., MATSUZAWA, T., YAGUCHI, T., YOSHIKAZU, H., NOVAKOVA, A., FRISVAD, J. C., TALBOT, J. J. & KOLARIK, M. 2018. Unravelling species boundaries in the *Aspergillus virdinutans* complex (section *Fumigati*): opportunistic human and anmal pathogens capable of interspecific hybridization. *Persoonia,* 41, in press, accepted for publication 14th March 2018.

JACOBSON, M. P., FRIESNER, R. A., XIANG, Z. & HONIG, B. 2002. On the role of crystal packing forces in determining protein sidechain conformations. *Journal of Molecular Biology,* 320**,** 597-608.

JACOBSON, M. P., PINCUS, D. L., RAPP, C. S., DAY, T. J. F., HONIG, B., SHAW, D. E. & FRIESNER, R. A. 2004. A hierarchical approach to all-atom protein loop prediction. *Proteins: Structure, Function and Bioinformatics,* 55**,** 351-367.

KIDD, S. E., GOEMAN, E., MEIS, J., F., SLAVIN, M. A. & VERWEIJ, P. E. 2015. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses,* 58**,** 350-355.

KIDD, S. E., WALKER, D., HAREMZA, E. & MORRIS, A. 2017. *Mycology QAP update* [Online]. The Royal College of Pathologies of Australia. Available: https://www.rcpa.edu.au/getattachment/25620d8b-928f-4552-86db-d5bcaeaad23b/Sarah-Kidd_Mycology-QAP-Update-Antifungal-Suscepti.aspx.

KUSUYA, Y., TAKAHASHI-NAKAGUCHI, A., TAKAHASHI, H. & YAGUCHI, T. 2015. Draft genome sequence of the pathogenic filamentous fungus *Aspergillus udagawae* strain IFM 46973T. *Genome Announcements,* 3**,** e00834-15.

LAVERGNE, R. A., MORIO, F., FAVENNEC, L., DOMINIQUE, S., MEIS, J. F., GARGALA, G., VERWEIJ, P. E. & LE PAPE, P. 2015. First description of azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation in France. *Antimicrobial Agents and Chemotherapy,* 59**,** 4331-5.

LI, J., ABEL, R., ZHU, K., CAO, Y., ZHAO, S. & FRIESNER, R. A. 2011. The VSGB 2.0 Model: A Next Generation Energy Model for High Resolution Protein Structure Modeling. *Proteins,* 79**,** 2794-2812.

LILLEHOJ, E. B. & CIEGLER, A. 1972. A toxic substance from *Aspergillus viridi-nutans*. *Canadian Journal of Microbiology,* 18**,** 193-197.

LILLEHOJ, E. B. & MILBURN, M. S. 1973. Viriditoxin production by *Aspergillus viridi-nutans* and related species. *Applied Microbiology,* 26**,** 202-205.

LIU, M., ZHENG, N., LI, D., ZHENG, H., ZHANG, L., GE, H. & LIU, W. 2016. cyp51A-based mechanism of azole resistance in *Aspergillus fumigatus*: Illustration by a new 3D Structural Model of *Aspergillus fumigatus CYP51A* protein. *Medical Mycology,* 54**,** 400-408.

LUTHY, R., BOWIE, J. U. & EISENBERG, D. 1992. Assessment of protein models with three-dimensional profiles. *Nature,* 356**,** 83-85.

LYSKOVA, P., HUBKA, V., SVOBODOVA, L., BARRS, V., DHAND, N. K., YAGUCHI, T., MATSUZAWA, T., HORIE, Y., KOLARIK, M., DOBIAS, R. & HAMAL, P. 2018. Antifungal susceptibility of the *Aspergillus viridinutans* complex: comparison of two *in vitro* methods. *Antimicrobial Agents and Chemotherapy,* 62.

MAVRIDOU, E., MELETIADIS, J., RIJS, A., MOUTON, J. W. & VERWEIJ, P. E. 2015. The strength of synergistic interaction between posaconazole and caspofungin depends on the underlying azole resistance mechanism of *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy,* 59**,** 1738-44.

MENDES, G., GONÇALVES, V. N., SOUZA-FAGUNDES, E. M., KOHLHOFF, M., ROSA, C. A., ZANI, C. L., COTA, B. B., ROSA, L. H. & JOHANN, S. 2016. Antifungal activity of extracts from Atacama Desert fungi against *Paracoccidioides brasiliensis* and identification of *Aspergillus felis* as a promising source of natural bioactive compounds. *Mem Inst Oswaldo Cruz.,* 111**,** 209-217.

MENEAU, I., COSTE, A. T. & SANGLARD, D. 2016. Identification of *Aspergillus fumigatus* multidrug transporter genes and their potential involvement in antifungal resistance. *Med Mycol,* 54**,** 616-27.

MIRCHEVSKA, G. & BOSSHARD, P. P. 2012. *In vitro* susceptibility testing of *Aspergillus* and non-*Aspergillus* filamentous moulds to antifungal agents: evaluation of three different methods *Journal of Antimicrobial Chemotherapy,* 5**,** 280-287.

MORTENSEN, K. L., JENSEN, R. H., JOHANSEN, H. K., SKOV, M., PRESSLER, T., HOWARD, S. J., LEATHERBARROW, H., MELLADO, E. & ARENDRUP, M. C. 2011. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis:

a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *Journal of Clinical Microbiology,* 49**,** 2243-51.

MORTENSEN, K. L., MELLADO, E., LASS-FLÖRL, C., RODRIGUEZ-TUDELA, J. L., JOHANSEN, H. K. & ARENDRUP, M. C. 2010. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrobial Agents and Chemotherapy,* 54**,** 4545-4549.

NARUSE, N., YAMAMOTO, H., MURATA, S., SAWADA, Y., FUKAGAWA, Y. & OKI, T. 1993. Aspochalasin E, a new antibiotic isolated from a fungus. *The Journal of Antibiotics (Tokyo),* 46**,** 679-681.

NASCIMENTO, A. M., GOLDMAN, G. H., PARK, S., MARRAS, S. A., DELMAS, G., OZA, U., LOLANS, K., DUDLEY, M. N., MANN, P. A. & PERLIN, D. S. 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrobial Agents and Chemotherapy,* 47**,** 1719-26.

NIWANO, Y., KUZUHARA, N., GOTO, Y., MUNECHIKA, Y., KODAMA, H., KANAI, K., YOSHIDA, M., MIYAZAKI, T. & YAMAGUCHI, H. 1999. Efficacy of NND-502, a novel imidazole antimycotic agent, in experimental models of *Candida albicans* and *Aspergillus fumigatus* infections. *International Journal of Antimicrobial Agents,* 12**,** 221-8.

PELÁEZ, T., ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., VALERIO, M., BLANCO, J. L., GARCIA, M. E., MUÑOZ, P., CUENCA-ESTRELLA, M. & BOUZA, E. 2013. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. *Journal of Medical Microbiology,* 62**,** 474-478.

POSTERARO, B., MATTEI, R., TRIVELLA, F., MAFFEI, A., TORRE, A., DE CAROLIS, E., POSTERARO, P., FADDA, G. & SANGUINETTI, M. 2011. Uncommon *Neosartorya udagawae* fungus as a causative agent of severe corneal infection. *J Clin Microbiol,* 49**,** 2357-2360.

SASTRY, G. M., ADZHIGIREY, M., DAY, T., ANNABHIMOJU, R. & SHERMAN, W. 2013. Protein and ligand preparation: Parameters, protocols, and influence on virtual screening enrichments. *Journal of Computer-Aided Molecular Design,* 27**,** 221-234.

SHARMA, C., HAGEN, F., MOROTI, R., MEIS, J. F. & CHOWDHARY, A. 2015. Triazole-resistant *Aspergillus fumigatus* harbouring G54 mutation: is it de novo or environmentally acquired? *Journal of Global Antimicrobial Resistance,* 3**,** 69-74.

SNELDERS, E., KARAWAJCZYK, A., SCHAFTENAAR, G., VERWEIJ, P. E. & MELCHERS, W. J. 2010. Azole resistance profile of amino acid changes in *Aspergillus fumigatus CYP51A* based on protein homology modeling. *Antimicrobial Agents and Chemotherapy,* 54**,** 2425-2430.

SUGUI, J. A., PETERSON, S. W., FIGAT, A., HANSEN, B., SAMSON, R. A., MELLADO, E., CUENCA-ESTRELLA, M. & KWON-CHUNG, K. J. 2014. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J Clin Microbiol,* 52**,** 3707-3721.

TALBOT JJ, HOUBRAKEN J, FRISVAD C, SAMSON RA, KIDD SE, PITT J, LINDSAY S, BEATTY JA & BARRS VR 2017. Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for animal and human fungal pathogens. *PLoS ONE,* 12**,** e0181660.

TALBOT, J. J. & BARRS, V. R. 2018. One-health pathogens in the Aspergillus viridinutans complex. *Medical Mycology,* 56**,** 1-12.

TALBOT, J. J., KIDD, S. E., MARTIN, P., BEATTY, J. A. & BARRS, V. R. 2015. Azole resistance in canine and feline isolates of *Aspergillus fumigatus*. *Comparative Immunology, Microbiology and Infectious Diseases,* 42**,** 37-41.

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A. & KUMAR, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution,* 30**,** 2725-2729.

ULLMANN, A. J., AGUADO, J. M., ARIKAN-AKDAGLI, S., DENNING, D. W., GROLL, A. H., LAGROU, K., LASS-FLORL, C., LEWIS, R. E., MUNOZ, P., VERWEIJ, P. E., WARRIS, A., ADER, F., AKOVA, M., ARENDRUP, M. C., BARNES, R. A., BEIGELMAN-AUBRY, C., BLOT, S., BOUZA, E., BRUGGEMANN, R. J. M., BUCHHEIDT, D., CADRANEL, J., CASTAGNOLA, E., CHAKRABARTI, A., CUENCA-ESTRELLA, M., DIMOPOULOS, G., FORTUN, J., GANGNEUX, J. P., GARBINO, J., HEINZ, W. J., HERBRECHT, R., HEUSSEL, C. P., KIBBLER, C. C., KLIMKO, N., KULLBERG, B. J., LANGE, C., LEHRNBECHER, T., LOFFLER, J., LORTHOLARY, O., MAERTENS, J., MARCHETTI, O., MEIS, J. F., PAGANO, L., RIBAUD, P., RICHARDSON, M., ROILIDES, E., RUHNKE, M., SANGUINETTI, M., SHEPPARD, D. C., SINKO, J., SKIADA, A., VEHRESCHILD, M., VISCOLI, C. & CORNELY, O. A. 2018. Diagnosis and management of *Aspergillus* diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline. *Clinical Microbiology and Infection,* 24 Suppl 1**,** e1-e38.

VAN DER LINDEN, J. W., CAMPS, S. M., KAMPINGA, G. A., ARENDS, J. P., DEBETS-OSSENKOPP, Y. J., HAAS, P. J., RIJNDERS, B. J., KUIJPER, E. J., VAN TIEL, F. H., VARGA, J., KARAWAJCZYK, A., ZOLL, J., MELCHERS, W. J. & VERWEIJ, P. E. 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clinical Infectious Diseases,* 57**,** 513-520.

VAN DER LINDEN, J. W., WARRIS, A. & VERWEIJ, P. E. 2011. *Aspergillus* species intrinsically resistant to antifungal agents. *Medical Mycology,* 49**,** S82-S89.

VAN INGEN, J., VAN DER LEE, H. A., RIJS, A. J., SNELDERS, E., MELCHERS, W. J. & VERWEIJ, P. E. 2015. High-Level Pan-Azole-Resistant Aspergillosis. *Journal of Clinical Microbiology,* 53**,** 2343-5.

VERWEIJ, P. E., CHOWDHARY, A., MELCHERS, W. J. G. & MEIS, J. F. 2016. Azole Resistance in Aspergillus fumigatus: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles? *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America,* 62**,** 362-368.

VINH, D. C., SHEA, Y. R., SUGUI, J. A., PARRILLA-CASTELLAR, E. R., FREEMAN, A. F., CAMPBELL, J. W., PITTALUGA, S., JONES, P. A., ZELAZNY, A., KLEINER, D.,

KWON-CHUNG, K. J. & HOLLAND, S. M. 2009. Invasive aspergillosis due to *Neosartorya udagawae*. *Clinical Infectious Diseases,* 49**,** 102-111.

WATTS, K. S., DALAL, P., MURPHY, R. B., SHERMAN, W., FRIESNER, R. A. & SHELLEY, J. C. 2010. ConfGen: a conformational search method for efficient generation of bioactive conformers. *Journal of Chemical Information and Modeling,* 50**,** 534-46.

WEBER, M., SCHAER, J., WALTHER, G., KAERGER, K., STEINMANN, J., RATH, P., SPIESS, B., BUCHHEIDT, D., HAMPRECHT, A. & KURZAI, O. 2017. FunResDB-A web resource for genotypic susceptibility testing of *Aspergillus fumigatus*. *Medical Mycology*.

WIEDERHOLD, N. P., GIL, V. G., GUTIERREZ, F., LINDNER, J. R., ALBATAINEH, M. T., MCCARTHY, D. I., SANDERS, C., FAN, H., FOTHERGILL, A. W. & SUTTON, D. A. 2016. First detection of TR34 L98H and TR46 Y121F T289A *Cyp51* mutations in *Aspergillus fumigatus* isolates in the United States. *Journal of Clinical Microbiology,* 54**,** 168-71.

WILLGER, S. D., PUTTIKAMONKUL, S., KIM, K. H., BURRITT, J. B., GRAHL, N., METZLER, L. J., BARBUCH, R., BARD, M., LAWRENCE, C. B. & CRAMER, R. A., JR. 2008. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *PLoS Pathogens,* 4**,** e1000200.

XIAO, L., LIU, H., WU, N., LIU, M., WEI, J., ZHANG, Y. & LIN, X. 2013. Characterization of the high cytochalasin E and rosellichalasin producing-*Aspergillus* sp. nov. F1 isolated from marine solar saltern in China. *World Journal of Microbiology and Biotechnology*, 29**,** 11-17.

ZARGARAN, M., TAGHIPOUR, S., KIASAT, N., ABOUALIGALEHDARI, E., REZAEI-MATEHKOLAEI, A., ZAREI MAHMOUDABADI, A. & SHAMSIZADEH, F. 2017. Luliconazole, an alternative antifungal agent against *Aspergillus terreus*. *Journal de Mycologie Medicale,* 27**,** 351-356.

Chapter 8. General Discussion

8.1 Introduction

The research in this thesis examined two threats to successful treatment of aspergillosis – infections with cryptic *Aspergillus* species and azole-resistant *Aspergillus fumigatus* (ARAf) isolates. It has investigated the prevalence of and certain virulence factors of pathogenic *Aspergillus* species causing disease in animals, including humans, examining both environmental and clinical isolates from Australia. This body of research further investigated the virulence factors of important one-health cryptic *Aspergillus* species in the *Aspergillus viridinutans* species complex (AVSC), and also investigated the prevalence of azole resistant *A. fumigatus* isolates in Australia.

8.2 Summary of Results

The work in this thesis produced the following novel findings:

1. Pathogenic, cryptic *Aspergillus* sect. *Fumigati* species are commonly isolated from residential environments in Australia.
2. There is an environmental niche for AVSC members, including pathogenic species (*A. felis*-clade), in natural habitats (soil) in Frankston, Victoria, Australia.
3. A novel species in section *Fumigati*, *A. frankstonensis*, belonging to the AVSC isolated from Frankston, Victoria.
4. Unique secondary metabolite profiles for *A. frankstonensis* environmental isolates including novel secondary metabolites, temporarily named DOLD, RAIMO and CALBO.
5. *A. fumigatus* is the most common cause of aspergillosis in captive birds in Australia.
6. *A. restrictus* is an aetiological agent of avian aspergillosis.
7. There is a low prevalence of azole resistance amongst environmental, veterinary and human isolates of *A. fumigatus* in Australia.
8. The *cyp51A* mutation F46Y in an azole resistant canine clinical *A. fumigatus* isolate from Australia from 1992.

9. The *cyp51A* mutations TR_{34}/L98H and G54R in azole resistant human clinical *A. fumigatus* isolates from Australia from 2015-2017.
10. Unique *cyp51A* gene DNA sequences of AVSC member species and that *cyp51A* is a useful gene for species' delimitation
11. *Cyp51A* gene mutations are not an important mechanism of azole resistance amongst AVSC member species.
12. *In vitro* antifungal susceptibility profiles of AVSC member species using CLSI and Sensititre™ YeastOne microbroth dilution testing.
13. Luliconazole (imidazole antifungal agent) returned low MIC values on CLSI antifungal susceptibility testing for AVSC isolates and is promising for treatment of triazole-resistant AVSC species infections.
14. Protein models for *cyp51A* single point mutations in *Aspergillus* species.

8.3 Further discussion

This thesis examined the prevalence of clinically relevant *Aspergillus* species in clinical (human and veterinary) and environmental (soil and air) samples from Australia. Notably, it further examined members of *A.* section *Fumigati*, and within this section, the AVSC and *A. fumigatus sensu stricto*. This led to the discovery of a new fungal species within the AVSC, a further understanding of the prevalence of pathogenic *A.* sect. *Fumigati* species in Australian environments and genus *Aspergillus* species in captive birds in Australia, the prevalence of ARAf in Australian environmental and clinical samples (human and veterinary) and reporting of AVSC antifungal susceptibilities and *cyp51A* amplification. Most AVSC species are known pathogens of humans and other animals (Vinh et al., 2009, Posteraro et al., 2011, Coelho et al., 2011, Gyotoku et al., 2012, Barrs et al., 2013, Peláez et al., 2013, Alvarez-Pérez et al., 2014, Sugui et al., 2014), so the discovery of another species is potentially clinically relevant. While the pathogenicity of this new species was not identified, its environmental discovery enhanced understanding of the niche of AVSC members. Additionally, CLSI *in vitro* antifungal susceptibility testing demonstrated high MICs to multiple azoles (itraconazole, voriconazole and isavuconazole) in *A. frankstonensis*, highlighting the potential for pathogenicity in this new species. Interestingly, *A. frankstonensis* was found with other known pathogenic AVSC members which also demonstrated high CLSI MICs of triazole therapeutics (Clinical and Laboratory Standards Institute (CLSI), 2018). The secondary metabolites of *A. felis* were also

examined, demonstrating potential pathogenicity in one isolate producing aszonapyrones, and both isolates producing chrysogine precursors (alkaloids). Several novel compounds also discovered will be examined in future work.

This study describes the *cyp51A* gene of AVSC species. Previously, AVSC clinical isolates were recognised as having higher MIC values of triazole antifungals than wild-type *A. fumigatus* isolates (Vinh et al., 2009, Posteraro et al., 2011, Gyotoku et al., 2012, Barrs et al., 2013, Peláez et al., 2013, Alvarez-Pérez et al., 2014, Sugui et al., 2014), and it was assumed this was an inherent mechanism of resistance rather than adaptive resistance due to *cyp51A* mutations as in ARAf, although no studies had investigated this. Additionally, previously the *cyp51A* gene had only been sequenced for two cryptic species in section *Fumigati*, *A. lentulus* and *A. udagawae* (Alcazar-Fuoli et al., 2011, Mellado et al., 2011, Kusuya et al., 2015). For *A. lentulus*, a similarity of 95% to wild-type *A. fumigatus cyp51A* was reported with 26 different amino acids observed between the two species, although no mutations were identified in the protein conserved regions (Alcazar-Fuoli et al., 2011, Mellado et al., 2011). For *A. udagawae*, the gene was sequenced as part of a draft genome project for this species and is not yet analysed (Kusuya et al., 2015). It was important to investigate this gene in other cryptic species. High MIC values of azole antifungals were found in most AVSC isolates tested, including environmental isolates. In comparison to the *cyp51A* gene of *A. fumigatus* species, many single point mutations were identified; however, the most common tandem repeat and polymorphisms associated with acquired ARAf were not identified. Difficulties in this process were deciphering between mutations that were non- functional that could be used as phylogenetic markers versus mutations conferring azole resistance. Very few mutations in the AVSC species were previously reported amongst ARAf isolates, with most being new mutations. This was overcome via an invaluable collaboration with pharmacists involving *cyp51A* protein modelling of the mutations unique to isolates with high MICs to azoles. This helped determine that these mutations were unlikely to confer azole resistance as they did not produce changes in the protein structure that would interfere with triazole drug binding to the haem. Thus, we identified that *cyp51A* mutations are not important in resistance in AVSC species. Instead, resistance may be innate in many AVSC isolates; or secondary, non-*cyp51A* mediated mechanisms, might confer resistance.

Although *cyp51A* mutations are most commonly reported in ARAf, they are not the only mechanism of azole resistance and it has been estimated that 10% of acquired azole resistance in *A. fumigatus* isolates is due to non-*cyp51A* mechanisms (Meis et al., 2016). Other mechanisms associated with resistance include the mutation P88L in the *HapE* gene, mutations and deletions in *afyap1, aldA, alga* and *AfCox10* genes, biofilm development, overexpression of adenosine triphosphate-binding cassette transporter (which has been linked to the TR_{34}/L98H mutation), cholesterol import mechanisms, overexpression of multidrug resistant efflux pump genes (*AfuMDR3* and *AfuMDR4*) and the transcriptional regulator *SrbA* (Nascimento et al., 2003, Willger et al., 2008, Camps et al., 2012, Paul et al., 2017). Mutations in *cyp51b* of *A. fumigatus* have also been investigated in connection with azole resistance (Mellado et al., 2001, Diaz-Guerra et al., 2003, Mellado et al., 2007, Buied et al., 2013). The two *cyp51A* genes in *Aspergillus fumigatus, cyp51A* and *cyp51B* both function in the biosynthesis of ergosterol and can be inhibited by azoles (Mellado et al., 2001, Mellado et al., 2007, Buied et al., 2013). An initial study looking at *cyp51A* and *cyp51B* sequence analysis in a few itraconazole resistant and susceptible isolates determined *cyp51B* mutations were less likely to play a role in azole resistance (Diaz-Guerra et al., 2003). The researchers discovered *cyp51A* and *cyp51B* single point mutations in three resistant isolates that led to amino acid changes, including G54E in *cyp51A* in all three isolates, Q42L and M236V in *cyp51B* in two of the isolates and D387E in *cyp51B* in one of the isolate. As the G54E mutation was common to all isolates and led to azole resistance when inserted into a wild-type isolate, it was concluded the *cyp51A* mutation was likely to be causing resistance in these isolates (Diaz-Guerra et al., 2003). Both *cyp51A* and *cyp51B* genes were amplified in a study examining resistance mechanisms in 14 azole resistant clinical isolates of *A. fumigatus* from human patients (Mellado et al., 2007). The TR_{34}/L98H mechanism was identified in all itraconazole resistant isolates. Additionally, two isolates also had *cyp51A* mutations S297T and F495I. No non-synonymous mutations were detected in the *cyp51B* gene in this study (Mellado et al., 2007). Another study examining 12 ARAf isolates with no *cyp51A* mutations found one isolate showed over induction of *cyp51B* following itraconazole exposure and another isolate had high *cyp51B* expression (Buied et al., 2013). No single point mutations were identified. The remaining 10 isolates had a similar induction of *cyp51B* to control wild-type isolates. It was proposed that overexpression and induction of *cyp51B* in the two isolates could cause azole resistance (Buied et al., 2013).

The commercially available Sensititre™ method is a reliable antifungal susceptibility test for determining MIC values in *A. fumigatus* species; however this thesis showed this method was less reliable at detecting azole resistance in AVSC isolates compared to the CLSI reference method. This is consistent with another recent study that also investigated EUCAST and Sensititre™ antifungal susceptibility testing on *A. hiratsukae, A. thermomutatus* and the AVSC member, *A. udagawae*, observing a low essential agreement for posaconazole of 67%; however for voriconazole and itraconazole the essential agreement for these three species was 100 % (Mello et al., 2017).

This thesis also examined AVSC susceptibility to the triazole drug isavuconazole. Isavuconazole is the most recently approved triazole drug for treatment of invasive aspergillosis. It has been recently tested for non-AVSC *Aspergillus* species isolates, with clinical breakpoints defined as 1 mg/L for *A. fumigatus* and *A. terreus*, and 0.25 mg/L for *A. nidulans.* The geometric mean MIC to isavuconazole for AVSC members tested in Chapter 7 (n=37) was higher than both these breakpoints for *Aspergillus* species at 2.96 mg/L (range 0.5-8.0 mg/L) (Denis et al., 2018). One previous study reported lower MICs to isavuconazole in 10 isolates of *A. udagawae* (geometric mean MIC 0.66 mg/L [range 0.25-1.0 mg/L] (Datta et al., 2013) compared to the seven *A. udagawae* isolates tested in this thesis (geometric mean MIC 2.21 mg/L [range 1.0-8.0 mg/L]); however the high MICs to isavuconazole amongst many AVSC isolates tested in this thesis suggests drugs with lower MIC values, such as luliconazole, should be further evaluated as better treatment options for AVSC member species.

This thesis also demonstrated close phylogenetic relationships between three species within the AVSC – *A. felis*, *A. pseudofelis* and *A. parafelis* (Barrs et al., 2013, Sugui et al., 2014). The preliminary work using multi-locus DNA phylogenetic analysis indicated that these three species are conspecific and may all be more appropriately designated *A. felis.* We suggested the use of *felis*-clade as an umbrella for *A. felis, A. parafelis* and *A. pseudofelis* while this was being resolved. A subsequent analysis of the AVSC preliminary results in support of the findings of this thesis were discussed in a recent publication (Lyskova et al., 2018). The phylogenetic status of *A. felis, A. parafelis* and *A. pseudofelis* as conspecific was confirmed in a comprehensive taxonomic study by Dr Vit Hubka at Charles University, Prague, which resolved *A. pseudofelis* and *A. parafelis* to be designated as *A.*

felis. My AVSC environmental isolates contributed to this collaborative investigation (Hubka et al., 2018).

While it was disappointing I did not find *A. felis,* the major pathogen of cats with sino-orbital aspergillosis, in the environment where infected cats resided, valuable information about the niche of these species in Australia was discovered, contributing to understanding the pathogenesis. It was shown that *A. felis* complex species could be isolated from surface soil in Frankston, Victoria, along with other closely related species, including the novel species discovered through my work, *A. frankstonensis*. This research and previous studies that isolated *A. felis* in the environment demonstrated these organisms are present in soil and dispersed through air (Barrs et al., 2013, Nováková et al., 2014). If samples were collected from domiciles with active infection a reservoir might have been located; however this could still be problematic as cats usually present with advanced chronic infection. Importantly, home environments were shown to be sources of other pathogenic *Aspergillus* species.

A lack of an environmental reservoir for *A. felis* when sampling may have been due to the following:

1. The soil reservoir that initiated infection was no longer present
2. The fungus was carried in by a mechanical vector e.g. insect or other animal species
3. The cats (all except one roamed their neighbourhoods) were exposed to the pathogen when roaming nearby
4. The cats were infected from an external source such as contaminated food or litter
5. The cats were infected elsewhere such as a boarding facility, veterinary hospital, breeder or other residence.

No common source of infection was identified when cat owners were surveyed regarding their cat's habits and husbandry. Properties of affected cats varied from rural, to semi-rural to urban and the cats varied from free-roaming to supervised outdoor access. Various litter substrates and food products were used with none common amongst infected cats. My initial testing

of food and litter types demonstrated *A. fumigatus* can grow on recycled paper pellets, so this could be a source of infection although *A. felis* was not isolated directly from this substrate, and not all cats used this type of litter. This research highlighted soil as a source of these fungal pathogens.

Previously, multi-locus molecular studies had not been undertaken on the cause of avian aspergillosis in Australia. Investigating this was interesting as captive birds are particularly susceptible to aspergillosis, usually due to high environmental burdens. It was important to investigate the aetiological agent given the high prevalence of cryptic *Aspergillus* species infecting other animals in Australia (Barrs et al., 2012, Barrs et al., 2013). From the avian study in this thesis, my previous undergraduate honours research on canine aspergillosis, investigations on feline aspergillosis and the various human epidemiology studies investigating molecular identification of pathogenic *Aspergillus* samples, it appears that cryptic *Aspergillus* species from the AVSC are only a common cause of aspergillosis in felines, only occasionally causing disease in other species, including dogs and humans. Interestingly this raises the question why are AVSC species infections so prevalent in felines compared to other species; is this due to increased environmental interactions between cats and the fungus, or immunological disorders of fungal clearance being more prevalent in cats, or a combination of these? Do cats have closer contact with a vector for fungal spores e.g. predation of contaminated hosts? Continued investigation into these aspects of host-pathogen-environment interactions is required. Immunological responses are being studied by my fellow University of Sydney PhD candidate, Dr Joanna Whitney, with differences observed between white blood cell populations in the nasal mucosa of affected cats compared to healthy controls, and observed differences in the inflammatory cell profile of affected cats compared to those produced by dogs and murine infection models mounting a protective acquired immunological response to invasive aspergillosis (Whitney et al., 2016).

This thesis demonstrates that *in vitro* testing of clotrimazole and enilconazole against *A. fumigatus* was effective at concentrations used for *in vivo* treatment of dogs with non-invasive SNA. As azole resistance was shown to be uncommon amongst canine isolates from Australia, Belgium and the USA from 1988 to 2014, topical and systemic therapies should be effective; however ongoing surveillance to detect ARAf is required. Treatment efficacy may be impacted by the technique used for application of topical treatment therapies, the number of treatments performed, and drug selection, dosage and length

of systemic treatments. While topical clotrimazole and enilconazole are the mainstays of therapy in dogs with SNA, with a higher success rate than systemic treatments (Sissener et al., 2006), topical therapy is contraindicated in dogs with evidence of cribriform plate lysis on computed tomography. However, a recent study of a few dogs (n=5) with sino-nasal aspergillosis and cribriform plate lysis with no neurological abnormalities, reported no adverse effects when the dogs were treated with topical clotrimazole, so this may not exclude this cohort from topical treatment. Four of the five dogs were followed up 2 years after treatment and owners reported no clinical signs of either SNA (e.g. nasal discharge, epistaxis) or neurological signs, although follow-up consultation with a veterinarian and diagnostic testing such as imaging was not performed (Stanton et al., 2018).

The investigation into azole resistance amongst canine isolates of *A. fumigatus* revealed a resistant phenotype in one Australian isolate which also harboured the *cyp51A* F46Y, G89E and E427K mutations. The F46Y and E427K have been reported in ARAf in humans and in susceptible *A. fumigatus* human derived isolates (Kidd et al., 2015). Investigating the F46Y mutation via *cyp51A* protein homology modelling in a model based on the *A. fumigatus cyp51B* crystal structure found this mutation was positioned at the substrate channel entrance concluding this could contribute to higher azole MIC values in isolates with these mutations, possibly by blocking the inhibitor entrance. The authors concluded this is likely a partial mechanism based on F46Y expression mutant modelling changes to azole susceptibility profiles (Garcia-Rubio et al., 2018). The same study found the E427K mutation resided in a non-conserved region at the protein surface, and thus had no interaction with azoles and no effect on the protein structure (Garcia- Rubio et al., 2018).

This thesis further investigated the prevalence of ARAf in Australia looking at the largest number of clinical and environmental isolates for the country. Previously there was little information on the prevalence of cryptic and pathogenic *Aspergillus* species in Australian environments, and ARAf in Australia (Kidd et al., 2015), with no environmental or veterinary ARAf studies. This was important to assess as ARAf is a major threat to effective treatment of aspergillosis in many other regions of the world, and where it has high prevalence, changes to treatment protocols are recommended (Verweij et al., 2015). Thus, appropriate disease management in Australia required more information on prevalence. These investigations revealed a low prevalence of ARAf amongst human and veterinary clinical isolates, with no evidence of ARAf amongst environmental isolates. Known *cyp51A* mutations were found in

three human clinical isolates, leading to the observed resistant phenotype. The Australian prevalence of ARAf of 2% among human clinical isolates is much lower than ARAf prevalence reports from Western Europe of up to 26%, and those reported from other Asia-Pacific member countries, including China, Japan, Pakistan and Taiwan of between 4.5% to 7.8% (Lockhart et al., 2011, Tashiro et al., 2012, Toyotome et al., 2016, Wu et al., 2015, Perveen et al., 2016). Importantly, this research indicates current international recommendations regarding the use of medical azoles in Australia do not need to be modified; however continued surveillance is recommended.

8.4 Future directions

Aspergillosis is a global health concern for humans and other animals. The Global Action Fund for Fungal Infections (GAFFI) estimates the global impact of fungal disease on humans. Based on data from 2005 to 2007, GAFFI estimates 1.37 million people are affected by chronic pulmonary aspergillosis, and that each year over 30 million people on corticosteroid therapy are at risk of invasive aspergillosis. GAFFI also reported that invasive aspergillosis has an annual infection rate of 300,000 people, with almost half in patients with chronic obstructive pulmonary disease, and in 2016, 56,000 AIDs patients died from aspergillosis (Global Action Fund for Fungal Infections (GAFFI), 2018). Thus, medical mycology is an important expanding scientific field with many opportunities for further research and collaboration. Since mycology employed polyphasic taxonomy, new *Aspergillus* species are discovered regularly, including opportunistic pathogens and isolates only discovered in the environment. Future studies could investigate the following:

1. Ongoing prospective multicentre surveillance of ARAf in clinical and environmental isolates in Australia.
2. *A. fumigatus cyp51A* crystal structure development for modelling of *cyp51A* gene alterations.
3. Sequencing of the *cyp51B* gene in AVSC member species to determine if mutations in this gene contribute to azole resistance in AVSC species.

4. Further analysis of secondary metabolites produced by AVSC species to further understand their potential pathogenicity.
5. Antifungal susceptibility testing and *cyp51* gene sequencing of a range of Genus *Aspergillus* species, including clinical and environmental isolates, to further understand the significance of mutations and the potential pathogenicity of newly discovered species
6. Development of novel antifungal therapies to circumvent azole resistance in *Aspergillus* species.
7. Further investigation of the potential for luliconazole treatment of AVSC infections.
8. Review agricultural industry practices regarding the application of antifungal drugs of the same class as medical antifungal therapies i.e. azole class drugs.
9. Regular screening for azole resistance amongst *A. fumigatus* environmental isolates where fungicidal azoles are used.
10. Environmental surveillance for AVSC members in cases of active infection in cats with aspergillosis due to these species.

8.5 Summary

Aspergillosis is a one-health infectious disease requiring input from multiple disciplines and collaborative studies to achieve better outcomes for human and veterinary patients. Continued research in this field will further our knowledge on the adaptability, virulence and niche living requirements of fungi, to better understand why they are such successful opportunistic pathogens and to exploit these factors to improve therapeutics. Globally, fungal pathogens affect millions of people annually. Antifungal resistance can no longer be ignored as the poor cousin of antibacterial resistance. I hope this thesis has demonstrated why fungi should not be ignored as a major player in the antimicrobial resistance threat to one health and the importance of correct fungal identification, antifungal susceptibility testing and *cyp51A* analysis of clinical and environmental isolates. This work reflects the importance of screening for both cryptic and azole-resistant *Aspergillus* species; one-health surveillance for both should continue.

8.6 References

ALCAZAR-FUOLI, L., CUESTA, I., RODRIGUEZ-TUDELA, J. L., CUENCA-ESTRELLA, M., SANGLARD, D. & MELLADO, E. 2011. Three-dimensional models of 14alpha-sterol demethylase (*Cyp51A*) from *Aspergillus lentulus* and *Aspergillus fumigatus*: an insight into differences in voriconazole interaction. *International Journal of Antimicrobial Agents,* 38**,** 426-34.

ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., BLANCO, J. L., GARCIA, M. E., KWON, M., MUÑOZ, P., CUENCA-ESTRELLA, M., BOUZA, E. & PELÁEZ, T. 2014. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. *Journal of Medical Microbiology,* 63**,** 617-619.

BARRS, V. R., HALLIDAY, C., MARTIN, P., WILSON, B., KROCKENBERGER, M., GUNEW, M., BENNETT, S., KOEHLMEYER, E., THOMPSON, A., FLIEGNER, R., HOCKING, A., SLEIMAN, S., O'BRIEN, C. & BEATTY, J. A. 2012. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *The Veterinary Journal,* 191**,** 58-64.

BARRS, V. R., VAN DOORN, T. M., HOUBRAKEN, J., KIDD, S. E., MARTIN, P., PINHEIRO, M. D., RICHARDSON, M., VARGA, J. & SAMSON, R. A. 2013. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One,* 8**,** e64871.

BUIED, A., MOORE, C. B., DENNING, D. W. & BOWYER, P. 2013. High-level expression of cyp51B in azole-resistant clinical *Aspergillus fumigatus* isolates. *Journal of Antimicrobial Chemotherapy,* 68**,** 512-4.

CAMPS, S. M., DUTILH, B. E., ARENDRUP, M. C., RIJS, A. J. M. M., SNELDERS, E., HUYNEN, M. A., VERWEIJ, P. E. & MELCHERS, W. J. G. 2012. Discovery of a *hapE* mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. *PLoS ONE,* 7**,** e50034.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) 2018. M59 Epidemiology cutoff values of antifungal susceptibility testing Clinical and Laboratory Standards Institute: 2nd ed. CLSI supplement M59, Wayne, PA.

COELHO, D., SILVA, S., VALE-SILVA, L., GOMES, H., PINTO, E., SARMENTO, A. & PINHEIRO, M. D. 2011. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. *Medical Mycology,* 49**,** 755-759.

DATTA, K., RHEE, P., BYRNES, E., 3RD, GARCIA-EFFRON, G., PERLIN, D. S., STAAB, J. F. & MARR, K. A. 2013. Isavuconazole activity against *Aspergillus lentulus, Neosartorya udagawae,* and *Cryptococcus gattii*, emerging fungal pathogens with reduced azole susceptibility. *Journal of Clinical Microbiology,* 51**,** 3090-3.

DENIS, J., LEDOUX, M. P., NIVOIX, Y. & HERBRECHT, R. 2018. Isavuconazole: A new broad-spectrum azole. Part 1: In vitro activity. *J Mycol Med,* 28**,** 8-14.

DIAZ-GUERRA, T. M., MELLADO, E., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2003. A point mutation in the 14alpha-sterol demethylase gene cyp51A contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy,* 47**,** 1120-4.

GARCIA-RUBIO, R., ALCAZAR-FUOLI, L., MONTEIRO, M. C., MONZON, S., CUESTA, I., PELAEZ, T. & MELLADO, E. 2018. Insight into the significance of *Aspergillus fumigatus cyp51A* polymorphisms. *Antimicrobial Agents and Chemotherapy,* 62**,** e00241-18.

GLOBAL ACTION FUND FOR FUNGAL INFECTIONS (GAFFI). 2018. *Fungi and Fungal Infections* [Online]. Available: https://www.gaffi.org/why/fungi-fungal-infections/ 2018].

GYOTOKU, H., IZUMIKAWA, K., IKEDA, H., TAKAZONO, T., MORINAGA, Y., NAKAMURA, S., IMAMURA, Y., NISHINO, T., MIYAZAKI, T., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., YASUOKA, A., YAGUCHI, T., OHNO, H., MIYZAKI, Y., KAMEI, K., KANDA, T. & KOHNO, S. 2012. A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. *Med Mycol.,* 50**,** 631-636.

HUBKA, V., BARRS, V., DUDOVA, Z., SKLENAR, F., KUBATOVA, A., MATSUZAWA, T., YAGUCHI, T., YOSHIKAZU, H., NOVAKOVA, A., FRISVAD, J. C., TALBOT, J. J. & KOLARIK, M. 2018. Unravelling species boundaries in the *Aspergillus virdinutans* complex (section *Fumigati*): opportunistic human and animal pathogens capable of interspecific hybridization. *Persoonia,* 41, in press, accepted for publication 14th March 2018.

KIDD, S. E., GOEMAN, E., MEIS, J., F., SLAVIN, M. A. & VERWEIJ, P. E. 2015. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses,* 58**,** 350-355.

KUSUYA, Y., TAKAHASHI-NAKAGUCHI, A., TAKAHASHI, H. & YAGUCHI, T. 2015. Draft genome sequence of the pathogenic filamentous fungus *Aspergillus udagawae* strain IFM 46973T. *Genome Announcements,* 3**,** e00834-15.

LOCKHART, S. R., FRADE, J. P., ETIENNE, K. A., PFALLER, M. A., DIEKEMA, D. J. & BALAJEE, S. A. 2011. Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the *cyp51A* gene. *Antimicrobial Agents and Chemotherapy,* 55**,** 4465-8.

LYSKOVA, P., HUBKA, V., SVOBODOVA, L., BARRS, V., DHAND, N. K., YAGUCHI, T., MATSUZAWA, T., HORIE, Y., KOLARIK, M., DOBIAS, R. & HAMAL, P. 2018. Antifungal susceptibility of the *Aspergillus viridinutans* complex: comparison of two *in vitro* methods. *Antimicrobial Agents and Chemotherapy,* 62.

MEIS, J. F., CHOWDHARY, A., RHODES, J. L., FISHER, M. C. & VERWEIJ, P. E. 2016. Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus*. *Philos Trans R Soc Lond B Biol Sci,* 371.

MELLADO, E., ALCAZAR-FUOLI, L., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2011. Role of *Aspergillus lentulus* 14-alpha sterol demethylase (*Cyp51A*) in azole drug susceptibility. *Antimicrobial Agents and Chemotherapy,* 55**,** 5459-68.

MELLADO, E., DIAZ-GUERRA, T. M., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2001. Identification of two different 14-alpha sterol demethylase-related genes

(*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *Journal of Clinical Microbiology,* 39**,** 2431-2438.

MELLADO, E., GARCIA-EFFRON, G., ALCAZAR-FUOLI, L., MELCHERS, W. J., VERWEIJ, P. E., CUENCA-ESTRELLA, M. & RODRIGUEZ-TUDELA, J. L. 2007. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. *Antimicrobial Agents and Chemotherapy,* 51**,** 1897-904.

MELLO, E., POSTERARO, B., VELLA, A., DE CAROLIS, E., TORELLI, R., D'INZEO, T., VERWEIJ, P. E. & SANGUINETTI, M. 2017. Susceptibility testing of common and uncommon *Aspergillus* Species against posaconazole and other mold-active antifungal azoles using the Sensititre method. *Antimicrobial Agents and Chemotherapy,* 61.

NASCIMENTO, A. M., GOLDMAN, G. H., PARK, S., MARRAS, S. A., DELMAS, G., OZA, U., LOLANS, K., DUDLEY, M. N., MANN, P. A. & PERLIN, D. S. 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrobial Agents and Chemotherapy,* 47**,** 1719-26.

NOVÁKOVÁ, A., HUBKA, V., DUDOVÁ, Z., MATSUZAWA, T., KUBÁTOVÁ, A., YAGUCHI, T. & KOLAŘÍK, M. 2014. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. *Fungal Diversity.,* 64**,** 253-274.

PAUL, S., DIEKEMA, D. & MOYE-ROWLEY, W. S. 2017. Contributions of both ATP-Binding Cassette Transporter and Cyp51A Proteins Are Essential for Azole Resistance in Aspergillus fumigatus. *Antimicrobial Agents and Chemotherapy,* 61**,** e02748-16.

PELÁEZ, T., ALVAREZ-PÉREZ, S., MELLADO, E., SERRANO, D., VALERIO, M., BLANCO, J. L., GARCIA, M. E., MUÑOZ, P., CUENCA-ESTRELLA, M. & BOUZA, E. 2013. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. *Journal of Medical Microbiology,* 62**,** 474-478.

PERVEEN, I., SEHAR, S., NAZ, I. & AHMED, S. Prospective evaluation of azole resistance in *Aspergillus fumigatus* clinical isolates in Pakistan. Seventh Advances Against Aspergillosis conference, 3-5 March 2016 2016 Manchester, UK.

POSTERARO, B., MATTEI, R., TRIVELLA, F., MAFFEI, A., TORRE, A., DE CAROLIS, E., POSTERARO, P., FADDA, G. & SANGUINETTI, M. 2011. Uncommon Neosartorya udagawae fungus as a causative agent of severe corneal infection. *J Clin Microbiol.,* 49**,** 2357-2360.

SISSENER, T. R., BACON, N. J., FRIEND, E., ANDERSON, D. M. & WHITE, R. A. 2006. Combined clotrimazole irrigation and depot therapy for canine nasal aspergillosis. *The Journal of Small Animal Practice,* 47**,** 312-5.

STANTON, J. A., MILLER, M. L., JOHNSON, P., DAVIGNON, D. L. & BARR, S. C. 2018. Treatment of canine sinonasal aspergillosis with clotrimazole infusion in patients with cribriform plate lysis. *Journal of Small Animal Practice*.

SUGUI, J. A., PETERSON, S. W., FIGAT, A., HANSEN, B., SAMSON, R. A., MELLADO, E., CUENCA-ESTRELLA, M. & KWON-CHUNG, K. J. 2014. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J Clin Microbiol.,* 52**,** 3707-3721.

TASHIRO, M., IZUMIKAWA, K., HIRANO, K., IDE, S., MIHARA, T., HOSOGAYA, N., TAKAZONO, T., MORINAGA, Y., NAKAMURA, S., KURIHARA, S., IMAMURA, Y., MIYAZAKI, T., NISHINO, T., TSUKAMOTO, M., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., YASUOKA, A., TASHIRO, T. & KOHNO, S. 2012. Correlation between triazole treatment history and susceptibility in clinically isolated *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy,* 56**,** 4870-5.

TOYOTOME, T., FUJIWARA, T., KIDA, H., MATSUMOTO, M., WADA, T. & KOMATSU, R. 2016. Azole susceptibility in clinical and environmental isolates of *Aspergillus fumigatus* from eastern Hokkaido, Japan. *Journal of Infection and Chemotherapy,* 22**,** 648-50.

VERWEIJ, P. E., ANANDA-RAJAH, M., ANDES, D., ARENDRUP, M. C., BRUGGEMANN, R. J., CHOWDHARY, A., CORNELY, O. A., DENNING, D. W., GROLL, A. H., IZUMIKAWA, K., KULLBERG, B. J., LAGROU, K., MAERTENS, J., MEIS, J. F., NEWTON, P., PAGE, I., SEYEDMOUSAVI, S., SHEPPARD, D. C., VISCOLI, C., WARRIS, A. & DONNELLY, J. P. 2015. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. *Drug Resistance Updates,* 21-22**,** 30-40.

VINH, D. C., SHEA, Y. R., SUGUI, J. A., PARRILLA-CASTELLAR, E. R., FREEMAN, A. F., CAMPBELL, J. W., PITTALUGA, S., JONES, P. A., ZELAZNY, A., KLEINER, D., KWON-CHUNG, K. J. & HOLLAND, S. M. 2009. Invasive aspergillosis due to *Neosartorya udagawae*. *Clinical Infectious Diseases,* 49**,** 102-111.

WHITNEY, J. L., KROCKENBERGER, M. B., DAY, M. J., BEATTY, J. A., DHAND, N. K. & BARRS, V. R. 2016. Immunohistochemical analysis of leucocyte subsets in the sinonasal mucosa of cats with upper respiratory tract aspergillosis. *Journal of Comparative Pathology,* 155**,** 130-140.

WILLGER, S. D., PUTTIKAMONKUL, S., KIM, K. H., BURRITT, J. B., GRAHL, N., METZLER, L. J., BARBUCH, R., BARD, M., LAWRENCE, C. B. & CRAMER, R. A., JR. 2008. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *PLoS Pathogens,* 4**,** e1000200.

WU, C. J., WANG, H. C., LEE, J. C., LO, H. J., DAI, C. T., CHOU, P. H., KO, W. C. & CHEN, Y. C. 2015. Azole-resistant *Aspergillus fumigatus* isolates carrying TR34/L98H mutations in Taiwan. *Mycoses,* 58**,** 544-9.

Chapter 9. Appendices

Appendix 1: One-health pathogens in the *Aspergillus viridinutans* complex

This is a pre-copy edited, author-produced version of an article accepted for publication in *Medical Mycology* following peer review. The version of record Talbot JJ, Barrs VR. One-health pathogens in the *Aspergillus viridinutans* complex, *Medical Mycology*, volume 56, issue 1, 1 January 2018, Pages 1–12, is available online at https://doi.org/10.1093/mmy/myx016.

ISHAM
INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2017, 0, 1–12
doi: 10.1093/mmy/myx016
Advance Access Publication Date: 0 2017
Review Article

Review Article

One-health pathogens in the *Aspergillus viridinutans* complex

Jessica J. Talbot and Vanessa R. Barrs*

The University of Sydney, NSW 2006 Australia, Faculty of Veterinary Science, School of Life and Environmental Sciences

*To whom correspondence should be addressed. Vanessa R. Barrs, BVSc(hons), PhD, MVetClinStud FANZCVS, University of Sydney, Sydney School of Veterinary Science, Faculty of Science, NSW 2006 Australia.
Tel: +61 2 9351 3437; Fax: +61 2 9351 7436; E-mail: vanessa.barrs@sydney.edu.au

Received 27 August 2016; Revised 13 December 2016; Accepted 21 February 2017; Editorial Decision 21 December 2016

Abstract

Cryptic species in *Aspergillus* section *Fumigati* are increasingly recognised as pathogens in humans and animals. The *A. viridinutans* complex (AVC) has recently expanded to comprise 10 species, of which six are known to be pathogenic, including *A. udagawae, A. felis, A. pseudofelis, A. parafelis, A. pseudoviridinutans,* and *A. wyomingensis*. They cause locally invasive and disseminated invasive disease syndromes, including chronic pulmonary aspergillosis and invasive aspergillosis in humans, invasive fungal rhinosinusitis in cats, and disseminated invasive aspergillosis in dogs. In contrast to *A. fumigatus*, AVC species are characterized by higher minimum inhibitory concentrations (MICs) of antifungal drugs and the infections they cause are typically more chronic and more refractory to therapy. This review, of relevance for one-health practitioners, explores the history of the AVC as well as current phylogenetic relationships, secondary metabolite production, environmental distribution, clinical syndromes, and antifungal susceptibility patterns.

Key words: Aspergillus, section Fumigati, Aspergillus viridinutans complex, cryptic species, aspergillosis.

Introduction

Aspergillus section *Fumigati* contains 63 species, 19 of which are known human and veterinary pathogens (Fig. 1).[1,2] Since the implementation of polyphasic taxonomical methods for fungal identification, there has been a striking increase in the number of cryptic and newly discovered species of medical importance within the section. Cryptic species can be defined as isolates of closely related fungal species that are indistinguishable on classical morphological features but can be identified to species level using molecular techniques.[3] The most common cryptic species in section *Fumigati* associated with clinical disease in humans are *A. lentulus*,[4–18] *A. thermomutatus*,[11,19,20] and members of the *A. viridinutans* complex (AVC).[6,21–26] Of the 10 species within this complex, six have been identified by multi-locus sequencing as pathogens in humans and animals including *A. udagawae, A. felis, A. pseudofelis, A. parafelis, A. pseudoviridinutans*, and *A. wyomingensis*. They cause locally invasive and disseminated invasive disease syndromes, have increased resistance to commonly used antifungal therapies, and are associated with high mortality rates.[4,21–27] Although *A. viridinutans sensu stricto* has been reported as

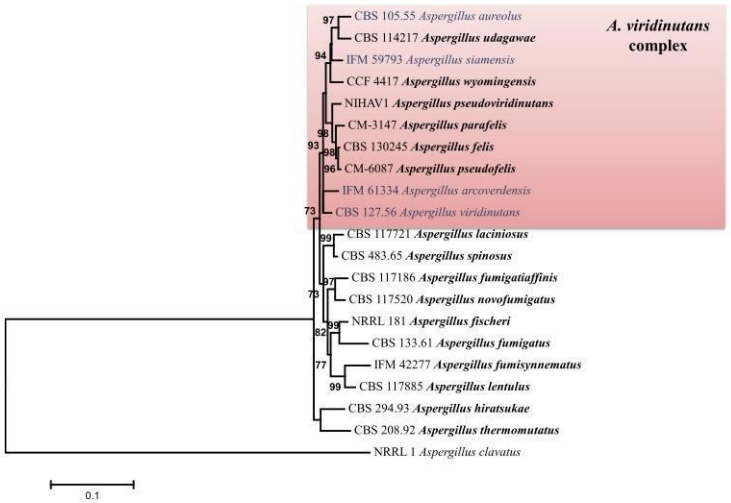


Figure 1. Phylogenetic tree depicting species in the *Aspergillus viridinutans* complex and pathogenic species in section *Fumigati* based on BenA and CaM DNA sequences of type strains.[4,54] All pathogenic species are set in bold. Nonpathogenic species are in blue font. Three pathogenic species in section *Fumigati*: *A. beijingensis, A. qizutongii,* and *A. wangduanlii* were not included since BenA and CaM sequence data are not available. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model[94] with 1,000 bootstrap replicates.[95] The tree with the highest log likelihood (−3539.8604) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches for values >70%. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories +*G*, parameter = 0.8578). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6.[96]

a pathogen, this is questionable as isolates in these reports were identified using morphological methods or only one gene was sequenced for phylogeny.[25–30] Some of these isolates have since been definitively identified and confirmed to be other species within the complex.[4,24,25,31] Three other species within the AVC, *A. aureoles, A. arcoverdensis,* and *A. siamensis* have been isolated from environmental samples only and their pathogenic potential is not known.[31–36] This review explores the history of the AVC and highlights the need for medical and veterinary practitioners to be aware of this important group of closely related fungi. Morphological features, secondary metabolite production, current phylogenetic relationships, clinical syndromes, and antifungal susceptibility patterns of the ten currently accepted members are discussed.

Species in the *A. viridinutans* complex and their characteristics

There are currently ten accepted species within the AVC: *A. viridinutans sensu stricto*, *A. udagawae*, *A. felis*, *A. wyomingensis*, *A. aureolus*, *A. parafelis*, *A. pseudofelis*, *A. pseudoviridinutans*, *A. siamensis,* and *A. arcoverdensis* (Fig. 1).[4,24,31,32,34–39] *Aspergillus viridinutans sensu stricto* was discovered in 1954 in the Australian environment.[39] This was shortly followed by the discovery of *Aspergillus aureolus,*[32] previously known as *Sartorya aureola*,[40] *Neosartorya aureola*[41] and *A. aureoluteus.*[42] It was not until the mid-1990s that another species, *Neosartorya udagawae* (now *A. udagawae*) was described and added to the complex.[34] In recent years the complex has further expanded. Since 2013 seven more species have been described, largely attributed to advances in polyphasic taxonomy, which utilizes morphologic, physiologic, and molecular characteristics to determine isolate speciation and phylogeny.[37,43]

Nomenclature changes

In 2011 the International Association for Plant Taxonomy (IAPT) adopted changes to the rules for naming fungi. A one fungus–one name approach was adopted, enforcing the adoption of the same genus name for teleomorph (sexual) and anamorph (asexual) types of a species.[44] This meant the sexual state of fungi previously known as *Neosartorya* was to be replaced with *Aspergillus*. Changes were also made around the Latin designation of a species epithet. The termination of a name is dependent on whether the

epithet is based on gender, geography, if there is an existing Latin name and species basionyms, as per Chapter III, Section 4 to Chapter IX, Section 2 of the International Code of Nomenclature for algae, fungi, and plants.[44] In cases where the names of species had been changed from their original name over time, names reverted to the original name. Thus, *Neosartorya udagawae* became *Aspergillus udagawae,* while *Aspergillus aureolus* maintained its basionym with no other variations.

Species in the AVC share some common morphological features. They are typically slow growing, slow to sporulate and thermophilic with optimal growth between 35° and 42°C.[4,24,31,32,34–39] *Aspergillus aureolus* and *A. siamensis* are the only homothallic species in the complex.[31,36]

Mating genotype and phenotype has been determined for *A. felis, A. wyomingensis, A. udagawae,* and *A. parafelis*.[4,24,31] Mating genotype, but not phenotype, has been confirmed in *A. viridinutans sensu stricto*, *A. pseudofelis, A. pseudoviridinutans,* and *A. arcoverdensis*.[4,31,35] Fertile interspecies matings have been reported between *A. parafelis* with *A. fumigatus sensu stricto, A. viridinutans sensu stricto, A. felis, A. pseudoviridinutans* and *A. wyomingensis*, and also between *A. pseudofelis* with *A. felis*.[4]

It is difficult to distinguish AVC species from other species in section *Fumigati* based on morphological features alone. Although members possess characteristic "nodding heads" on cytological examination (Fig. 2), this feature occurs in some other section *Fumigati* species.[31] The inability to grow at 50°C can be exploited as a practical method in clinical laboratories to distinguish cryptic species in section *Fumigati* from *A. fumigatus sensu stricto*.[4,24,31] Distinguishing between species within the AVC is also difficult based on anamorph and teleomorph morphological features. The gold standard for identification is through the addition of molecular identification to morphological techniques.

Molecular identification

Phylogenetic relationships within the AVC have been determined by comparative sequence analysis of multiple loci including the internal transcribed spacer region (*ITS*), beta-tubulin (*benA*), calmodulin (*caM*), rodlet A (*rodA*), minichromosome maintenance factor (*Mcm7*), a pre-RNA processing protein (*Tsr1*), and the second largest unit of RNA polymerase II (*RPB2*).[4,24,31,42,43,45–49] Comparative multilocus sequence analysis is the gold standard for molecular identification although no one gene is currently accepted as a stand-alone method for identification. [33,38] The most commonly used genes that have been used for species descriptions are *benA* and *caM*. [4,24,31,35,36]

Figure 2. Nodding conidial head typical of *Aspergillus viridinutans* complex species. *A. parafelis* isolate ×100 light microscopy image.

Secondary metabolites

Determination of secondary metabolites may be useful to predict pathogenicity where only environmental isolates exist for a species.[1] Viriditoxin is a secondary metabolite produced by all species in the AVC.[1,50] The role of viriditoxin in disease pathogenesis is unknown, since it is produced by both known pathogens and species of unknown pathogenicity. However, one study demonstrated lethality in mice following intraperitoneal administration (LD_{50} of 2.8 mg), as well as the ability to inhibit growth of several bacterial species (*Corynebacterium, Bacillus,* and *Streptococcus*).[50,51] Viriditoxin is also produced by one other species in section *Fumigati*, *Aspergillus denticulate*.[52] *Aspergillus brevipes* was also reported to produce viriditoxin; however, this was since confirmed to be present in a mixed culture with *A. viridinutans sensu stricto*.[37,53,54]

A number of novel bioactive compounds have been discovered through secondary metabolite extraction of AVC species. Some extrolites have demonstrated anti-cancer and antibacterial properties. [50,55–58] Recent investigations into secondary metabolite extracts from environmental *A. felis*

isolates demonstrated *in vitro* activity against *Paracoccidiodes brasiliensis PB*18.[58] Extracts from a range of fungal genera were tested, with isolates of *A. felis* and *A. udagawae* demonstrating the greatest activity against *P. brasiliensis PB*18. Using reversed-phase HPLC, *A. felis* extracts were identified as pyripyropene A, aspochalasin E, and cytochalasins (rosellichalasin, cytochalasin Kasp, and cytochalasin E) as well as a number of unknown compounds. Rosellichalasin and cytochalasin E have proven cytotoxic activity against human tumour cells lines,[59] while aspochalasin E is active against the cancer cells murine melanoma B16-F10 and human colon carcinoma HCT-116 cells.[60] Secondary metabolites produced from clinical and environmental isolates of both *A. udagawae* and *A. viridinutans sensu stricto* include fumigaclavine C, helvolic acid, methyl-sulochrin, pyripyropene A and E and trypacidin. *Aspergillus udagawae* also produces fumigaclavine A and fumiquinazoline F/G, while *A. viridinutans sensu stricto* also produces fumagillin, fumitremorgins A and C, and pyripyropenes O and S.[61] However, in the study that characterized these secondary metabolites, the clinical isolates listed with *BenA* and *CaM* sequences available on GenBank[62] (*A. udagawae* IFM 5058, IFM 51744, IFM 53867, IFM 53868, IFM 54303; *A. viridinutans* IFM 54303), only had identity similarities with *BenA* and *CaM* markers for *A. udagawae* (AF132226, AB748566) and *A. viridinutans sensu stricto* (AF134779, DQ534162)[38] type strains of 97–99%.[61] Therefore, additional comparative gene sequence analyses may be necessary for definitive species identification of these isolates.

Recently MALDI-ToF mass spectrometry has been utilized for rapid identification of *Aspergillus* sp., including differentiation of *A. fumigatus sensu stricto* from the cryptic species *A. lentulus*.[63] More work is required for accurate identification of species within the AVC. However, this is a promising technique that is already used in clinical microbiology laboratories worldwide.

Identification of isolates in the clinical microbiology laboratory

Given the clinical management implications, clinical microbiologists can utilize specific laboratory techniques to increase recognition of cryptic species in section *Fumigati*. Knowledge of the correct fungal species is clinically relevant due to *in vitro* and *in vivo* antifungal resistance to commonly used therapeutics associated with some cryptic species infections.[7,8,19,64,65] Accurate identification is also essential for epidemiological studies to ascertain the prevalence of cryptic species infections in hospital populations.[19,64,65] While polyphasic taxonomy is the gold standard for species delimitation, this is not practical in the time crucial setting of clinical disease. Accurate identification of clinical isolates causing aspergillosis can be achieved using comparative sequence analysis of the partial *BenA* and *CaM* genes in laboratories where molecular techniques are available.[4,24,31,35,36] Initial isolation on malt extract agar at 37°C is suggested as these species will generally sporulate within 7 days on this medium for microscopic analysis and DNA extraction.[24] The presence of a 'nodding head' (Fig. 2) should alert clinical mycologists to the possibility of an AVC species; however, other section *Fumigati* isolates have also been reported to share this feature.[31] The incubation of an isolate at 50°C is usually sufficient to distinguish most isolates of *A. fumigatus,* which is thermoresistant, from AVC species, which generally do not grow at temperatures higher than 42–45°C.[3,24,31,32,34–39] Slow sporulation of an isolate and high MIC values of antifungals should alert clinicians and laboratory mycologists to the possibility of cryptic species infection, and trigger molecular testing for definitive species identification. In addition to comparative sequence analysis, multiplex polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) are other molecular techniques with potential application for identification of clinical isolates.[6,66,67]

Environmental prevalence

Aspergillus viridinutans complex species have been isolated from a range of habitats, and most appear to have worldwide distribution, including North and South America, Europe, Africa, Northeast Asia, and Asia-Pacific (Table 1). Four species in the AVC (*A. arcoverdensis, A. aureolus, A. siamensis* and *A. viridinutans sensu stricto*) have only been found in the environment and are of unknown pathogenic status.[32,34–36,39,68] The other six species have been isolated from both environmental and clinical samples. Environmental isolates have been sourced predominantly from soil, as well as food, air, animal feces, and desert rocks. *Aspergillus felis* has been isolated from a variety of substrates, reflecting the ability of this organism to survive in a range of environments, and it appears to be globally distributed.[24,31,55] To date only small numbers of *A. parafelis, A. pseudofelis,* and *A. pseudoviridinutans* have only been reported, and their environmental distribution is unknown (Table 1). *Aspergillus arcoverdensis* has been isolated from arid, semi-desert soil, and forest soil, demonstrating widespread distribution and an ability to survive in diverse environments.[35] The diverse range of environments inhabited by members of this complex, coupled with their potential for virulence, highlights the importance for medical and veterinary practitioners to be aware of these species.

Table 1. Environmental *Aspergillus viridinutans* complex isolates.

Species	Sources	Geographic region	Publications
A. arcoverdensis ($n = 11$)	Semi-desert soil	Brazil ($n = 11$)	Matsuzawa et al. 2015[35]
A. aureolus ($n = 6$)	Soil	Ghana ($n = 2$), Liberia ($n = 1$), Brazil ($n = 2$), unknown ($n = 1$)	Fennell and Raper 1955[32]; Raper and Fennell 1965[33]; Horie et al. 1995[34]; Novakova et al. 2014[31]
A. felis ($n = 12$)	Indoor air Soil Food	Germany ($n = 1$), USA ($n = 4$), Sri Lanka (n = 2), India ($n = 1$), Czech Republic ($n = 1$), Japan ($n = 1$),	Barrs et al. 2013[24]; Novakova et al. 2014[31]; Gonçalves et al. 2016[55]
	Pinus caribea Rocks	Chile (n = 1), Zambia (n = 1)	
A. siamensis ($n = 3$)	Coastal forest soil	Thailand ($n = 3$)	Eamvijarn et al. 2013[36]
A. udagawae ($n = 57$)	Soil Rocks	USA ($n = 53$), Brazil ($n = 3$) Chile ($n = 1$)	Hong et al. 2010[93]; Horie et al. 1995[34]; Novakova et al. 2014[31]; Gonçalves et al. 2016[55]
A. viridinutans sensu stricto (n = 3)	Rabbit dung Sandy podzol Soil	Australia ($n = 3$)	McLennan et al. 1954[39] Katz et al. 2005[28,a]
A. wyomingensis (n = 14)	Soil	USA ($n = 12$) China ($n = 1$) Russia ($n = 1$)	Novakova et al. 2014[31] Varga et al. 2000[66]

[a]Identification questionable: nucleotide homology < 99% with type strain sequence.

Clinical disease and antifungal resistance

Cases of aspergillosis due to AVC species are being increasingly reported amongst human and veterinary patients, reflecting the importance of a One-Health approach to these mycoses (Table 2). Six species are recognized as pathogens: *A. udagawae*, *A. felis*, *A. wyomingensis*, *A. pseudofelis*, *A. parafelis*, and *A. pseudoviridinutans*.[2,4,21,24] Previous reports included *A. viridinutans sensu stricto* as a cause of disease in people; however, multilocus molecular analysis of these strains revealed they were *A. felis*, *A. parafelis*, *A. pseudofelis*, or *A. pseudoviridinutans*.[4,24–27]

These infections fall within the scope of disease due to 'cryptic' *Aspergillus* species in section *Fumigati*. In recent studies where multilocus comparative sequence analysis was used for identification, the prevalence of cryptic *Aspergillus sp*. infections in section *Fumigati* among human patients was 3–5%.[8,19,64] One European study examined 162 *Aspergillus* section *Fumigati* strains from hospitals in Spain and identified six cryptic section *Fumigati* species causing aspergillosis (3.7%), including one AVC species.[64] The Transplant-Associated Infection Surveillance Network and the American Centers for Disease Control and Prevention, Atlanta investigated cryptic species prevalence among transplant recipients with invasive aspergillosis ($n = 216$) and showed that 68% of isolates were from section *Fumigati*, amongst which there was a cryptic species prevalence of 5%. *Aspergillus udagawae* caused 2% of section *Fumigati* infections overall, and 37.5% of cryptic *A.* section *Fumigati* sp. infections.[8] Another study of 133 *Aspergillus sp*. isolates from human patients in Brazil showed 19% were cryptic species in section *Fumigati* but none belonged to the AVC.[19]

In section *Fumigati*, cryptic species infections in humans are most commonly caused by *A. lentulus*,[4–18] *A. udagawae*,[6,21–23] *A. thermomutatus*,[11,19,20] and *A. felis*.[24,25] Two other species *A. fumigatiaffini*[11] and *A. novofumigatus*[14,26] have been isolated from clinical specimens, however futher evidence to support their role as pathogens is required. Further prevalence data are also needed in the veterinary field, since there are few molecular based studies. One study found no cryptic species in section *Fumigati* in dogs with noninvasive fungal rhinosinusitis, which is the most common anatomic form of aspergillosis in this host.[69] This is in contrast to the high prevalence of cryptic section *Fumigati* species isolated from cases of fungal rhinosinusitis in cats (72%, $n = 33/46$), of which the majority (81%, $n = 29/36$) were AVC species.[2,24,70–74] Clinical disease in dogs and cats occurs most commonly in systemically immunocompetent animals. Information gleaned from naturally infected animal hosts can be used to further knowledge across human and veterinary medicine, including agents of disease, their virulence, antifungal susceptibilities, disease progression, and management.

Table 2. Published clinical cases caused by *Aspergillus viridinutans* complex species where molecular sequencing techniques were used in addition to morphological identification.[a]

Fungal species	Host	Origin	Source	Publication	Gene sequenced
A. felis ($n = 14$)	Cat	Australia	Retrobulbar mass	Barrs et al. 2013[24] Katz et al. 2005[28]	*ITS, BenA, CaM Alkaline protease, BenA, ITS*
A. felis ($n = 1$)	Dog	Australia	Vitreous humor	Barrs et al. 2013[24]	*ITS, BenA, CaM*
A. felis ($n = 1$)	Cat	UK	Retrobulbar mass	Barrs et al. 2013[24]	*ITS, BenA, CaM*
A. felis ($n = 1$)	Cat	Australia	Thoracic mass	Barrs et al. 2013[24]	*ITS, BenA, CaM*
A. felis ($n = 1$)	Cat	Australia	Sino-nasal cavity	Barrs et al. 2013[24]	*ITS, BenA, CaM*
A. felis ($n = 1$)	Human	Spain	Sputum, BAL	Barrs et al. 2013[24] Pelaez et al. 2013[26]	*ITS, BenA, CaM*
A. felis ($n = 1$)	Human	Portugal	Lung	Barrs et al. 2013[24] Coelho et al. 2011[27]	*ITS, BenA, CaM, rodA*
A. felis ($n = 1$)	Human	Japan	Thigh bone abscess	Novakova et al. 2014[88]	*BenA, CaM*
A. parafelis ($n = 1$)	Human	Portugal	Lung	Sugui et al. 2014[4]	*BenA, CaM, MCM7, RPB2, TSR1*
A. parafelis ($n = 1$)	Human	Spain	Oropharyngeal exudate	Sugui et al. 2014[4] Alcazar-Fuoli et al. 2008[11]	*BenA, CaM, MCM7, RPB2, TSR1, rodA*
A. pseudofelis ($n = 1$)	Human	Spain	Nail	Sugui et al. 2014[4] Alcazar-Fuoli et al. 2008[11]	*BenA, CaM, MCM7, RPB2, TSR1, rodA*
A. pseudofelis ($n = 1$)	Human	Spain	Sputum	Sugui et al. 2014[4]	*BenA, CaM, MCM7, RPB2, TSR1*
A. pseudoviridinutans ($n = 1$)	Human	USA	Lymph node	Sugui et al. 2014[4] Vinh et al. 2009[29]	*BenA, CaM, MCM7, RPB2, TSR1*
A. udagawae ($n = 1$)	Human	Japan	BAL fluid	Gyotoku et al. 2012[22]	*BenA*[a]
A. udagawae ($n = 1$)	Human	USA	BAL fluid	Farrell et al. 2014[75]	*ITS, BenA, CaM*
A. udagawae ($n = 1$)	Human	Italy	Cornea	Posteraro et al. 2011[23]	*ITS, BenA, CaM*
A. udagawae ($n = 4$)	Human	USA	Lung	Vinh et al. 2009[21]	*ITS, BenA, rodA*
A. udagawae ($n = 1$)	Human	Czech Republic	Nail	Novakova et al. 2014[31]	*CaM*[a]
A. udagawae ($n = 2$)	Human	USA	Lung	Sugui et al. 2010[76]	*BenA, rodA*
A. udagawae ($n = 3$)	Human	Japan	Unknown	Tamiya et al. 2015[61]	*BenA, CaM*
A. udagawae ($n = 1$)	Human	Japan	BAL fluid	Tamiya et al. 2015[61]	*BenA, CaM*
A. udagawae ($n = 1$)	Human	Japan	Ocular bulb	Tamiya et al. 2015[61]	*BenA, CaM*
A. udagawae ($n = 3$)	Human	USA	BAL fluid	Balajee et al. 2006[6]	*BenA, rodA, RFLP*[a]
A. udagawae ($n = 1$)	Human	USA	Lung	Balajee et al. 2006[6]	*BenA, rodA, RFLP*[a]
A. udagawae ($n = 2$)	Human	Unknown	Unknown	Balajee et al. 2006[6]	*BenA, rodA, RFLP*[a]
A. udagawae ($n = 1$)	Human	Unknown	Brain	Balajee et al. 2006[6]	*BenA, rodA, RFLP*[a]
A. udagawae ($n = 2$)	Human	Spain	Unknown	Escribano et al. 2013[14]	*BenA*[a]
A. udagawae ($n = 1$)	Cat	Australia	Sinonasal cavity	Barrs et al. 2014[2]	*BenA, CaM*
A. udagawae ($n = 1$)	Cat	Australia	Respiratory tract	Barrs et al. 2013[24]Katz et al. 2005[28]	*ITS, BenA, CaM Alkaline protease, BenA, ITS*
A. udagawae ($n = 1$)	Cat	USA	Retrobulbar mass	Barrs et al. 2014[2]	*CaM*[a]
A. udagawae ($n = 2$)	Cat	Japan	Retrobulbar mass	Kano et al. 2008, 2013[72,73]	*ITS, BenA* [a]
A. udagawae ($n = 3$)	Human	USA	Lung	Balajee et al. 2009[8]	*ITS, BenA*[a]
A. viridinutans ($n = 1$)	Human	Japan	Cornea	Shigeyasu 2012[30]	*BenA*[a]
A. viridinutans ($n = 1$)	Human	Japan	Unknown	Tamiya et al. 2015[61]	*BenA, CaM*[a]
A. viridinutans ($n = 1$)	Human	Japan	Lung	Tamiya et al. 2015[61]	*BenA, CaM*[a]
A. viridinutans ($n = 1$)	Human	USA	Lung	Vinh et al. 2009[29]	*ITS, BenA, rodA*[a]
A. viridinutans ($n = 1$)	Human	Spain	Unknown	Alastruey-Izquierdo et al. 2013[64]	*ITS, BenA*[a]
A. viridinutans ($n = 1$)	Human	Spain	Unknown	Escribano et al. 2013[14]	*BenA*[a]
A. viridinutans ($n = 1$)	Human	Norway	Brain	Andersen et al. 2012[77]	*ITS*[a]
A. wyomingensis ($n = 1$)	Cat	Australia	Retrobulbar mass	Barrs et al. 2014[2]	*ITS, BenA*

[a]Identification questionable: nucleotide homology <99% with type strain sequence, no GenBank sequence available or only one gene sequenced.
BAL = Bronchoalveolar lavage.

Human infections

Human infections caused by the AVC have been reported in Spain, Portugal, the Czech Republic, Italy, Norway, the United States, and Japan. The most common presentation is chronic invasive pulmonary aspergillosis in immunosuppressed patients, which can become disseminated.[4,21,24,26,27] *Aspergillus felis*, *A. parafelis*, and *A. udagawae* have been isolated from such cases.[4,21,24] One patient with leukemia and *A. felis* infection was coinfected with *A. novofumigatus*.[26] Another case, caused by *A. parafelis*, was characterized by chronic spread of infection across anatomical planes, including cervical and thoracic lymph nodes, lungs, and pleura.[4,27] In addition, invasive pulmonary aspergillosis due to *A. udagawae* has a more chronic disease course than that caused by *A. fumigatus sensu stricto*.[21] *Aspergillus udagawae* has also been reported to cause localised bronchial infection in an elderly immunosuppressed patient,[22] acute respiratory distress syndrome (ARDS) in a middle-aged immunocompetent patient,[75] and a severe corneal infection.[23] In a study investigating the prevalence of cryptic species among US and European reference laboratory collections of isolates morphologically identified as *A. fumigatus*, RFLP analysis, and multilocus sequence typing identified *A. udagawae* from brain, sputum, BAL, and lung samples.[11]

Other clinical isolates obtained from patients include *A. pseudofelis* from sputum and a nail, *A. parafelis* from oropharyngeal exudates, *A. pseudoviridinutans* from a mediastinal lymph node,[4] and *A. udagawae* from a nail.[31] The identity of some clinical isolates, reported to be *A. udagawae* or *A. viridinutans sensu stricto*, is likely to be inaccurate for at least some isolates, since some were based on nucleotide homology of <99% with available, most closely related type strain sequences, or only one gene region was sequenced or the sequence was not submitted to GenBank.[62] Such isolates require further identification, especially in light of the number of recently discovered new species in the *A. viridinutans* complex.[6,14,21,29,61,64,76–77]

Animal infections

The most commonly described syndrome caused by the AVC is sino-orbital aspergillosis (SOA) in cats, a form of chronic invasive fungal rhinosinusitis. Disease has been reported in three geographical regions: Europe, Asia, and Australia.[24,71–73] Infection is most commonly caused by *A. felis*, but *A. udagawae* and *A. wyomingensis* have also been isolated from affected cats.[2,24,72,73] While *A. viridinutans sensu stricto* was reported as a cause of feline infection, this is doubtful as its identity was based on sequencing of the partial beta-tubulin gene only and the closest match using the GenBank BLAST tool[62] was 99%.[73] Previous isolates showing a similar percentage homology to other members of this complex have been demonstrated to be different species.[4,24,31,71]

Feline SOA carries a poor prognosis and is often fatal.[24,70,71] Cats typically present with signs of an orbital mass (exophthalmos, prolapse of the nictitating membrane (third eyelid), exposure keratitis and conjunctival hyperaemia), and signs or a recent history of nasal disease (sneezing, nasal discharge, epistaxis). Extension of disease beyond the orbit often results in an oral mass or ulcer in the pterygopalatine fossa and paranasal soft tissue swelling. Mild mandibular lymphadenopathy is also common. In advanced disease, neurological signs including hyperaesthesia and seizures may be present.[24,70,71] Infection due to A. *felis* has also been reported in a dog with disseminated invasive aspergillosis.[24]

It is currently unclear why *A. felis* is a major fungal pathogen of cats but is rarely involved in human or canine aspergillosis. Interestingly, in cats infection occurs in apparently systemically immunocompetent individuals, whereas human and canine infections have occurred in immunocompromised patients.[2] Around 40% of affected cats are pure-breeds of Persian lineage and have a brachycephalic facial conformation with foreshortening of the skull and anterior rotation of the nasal bones.[70] Infection may be more likely in cats due to an environmental exposure factor (e.g., their exposure to fungal spores in the environment may be secondary to a particular feline behaviour such as burying feces) and/or genetic factors such as a deficient innate immune response (e.g., problems with local immune factors).[78]

Antifungal therapy

The current recommended primary therapy for invasive aspergillosis in humans is voriconazole, with isavuconazole and amphotericin B acceptable as alternatives for treatment failures.[79] Given AVC species can have high MICs of voriconazole,[4,21–26] patient management can be greatly impacted by the failure to recognise the etiological agent, leading to treatment failure and protracted illness. Therefore, correct identification of the species causing invasive aspergillosis is crucial for optimal patient treatment.

The optimal treatment for cats with SOA caused by *A. viridinutans* complex species is yet to be identified. Most treated cats have been euthanased due to disease progression despite aggressive antifungal therapy using itraconazole or posaconazole as monotherapy or combined with amphotericin B, or with amphotericin B and terbinafine.[71] A benefit of adding exenteration to the treatment regime

has not been demonstrated. Voriconazole can have serious adverse effects on cats and cannot be used for first line therapy.[71,80,81] The echinocandin caspofungin was effective in one case that failed therapy with triazoles and amphotericin B.[71]

Antifungal resistance

Azole resistance amongst *Aspergillus species* is an increasing global problem and can be primary (inherent) or secondary (acquired). Mutations in the Cyp51A gene are associated with secondary azole resistance amongst human clinical isolates of *A. fumigatus*.[82–85] Cyp51A mutations have not yet been investigated in AVC species. The draft genome sequence of *A. udagawae* has been recently released and may help identify regions that contribute to resistance mechanisms.[86]

Resistance mechanisms have been investigated for another cryptic *A.* section *Fumigati* species, *A. lentulus*.[87,88] These studies concluded that the Cyp51A mechanisms conferring resistance for *A. lentulus* differ from that of *A. fumigatus*. A three-dimensional protein model was used to explore interactions between Cyp51A and voriconazole.[88] When *A. fumigatus* and *A. lentulus* cyp51A proteins were compared, a difference in the BC loop of the protein led to differences in the BC loop lock-up of voriconazole, which could account for differences in antifungal susceptibility profiles between these two species. The prevalence of acquired versus inherent resistance mechanisms amongst AVC species is unknown. Epidemiological cutoff values (ECVs) for antifungal susceptibility testing have not yet been established for this complex. The MICs of all classes of antifungal drugs are often higher in AVC species compared to ECVs established for *A. fumigatus* (Table 3).[89–91] *In vitro* and *in vivo* resistance to triazoles in human patients with AVC infections, have resulted in treatment failure.[6,11,21,23,26,27] One patient with chronic invasive pulmonary aspergillosis due to *A. parafelis* infection failed to respond to itraconazole initially, had a partial response to posaconazole, then relapsed during voriconazole therapy.[4,27] Combination therapy with posaconazole and caspofungin was also unsuccessful, and the patient died. Antifungal susceptibility testing of the infecting *A. parafelis* isolate obtained at post mortem demonstrated high MIC values of voriconazole (4 μg/ml) and itraconazole (>16 μg/ml).[4,27]

Another patient with leukaemia and invasive pulmonary aspergillosis due to *A. felis* was treated using combination therapy with voriconazole, caspofungin and amphotericin B. The patient had a mixed infection with other *Aspergillus* sp. (initially *A. calidoustus* and *A. novofumigatus,* then *A. novofumigatus* and *A. felis*) and died. All infecting isolates had high MICs of voriconazole (4–8 μg/ml).[24–26]

High MICs of amphotericin B, itraconazole and voriconazole have been observed among *A. udagawae* isolates from clinical infections when compared to *A. fumigatus*, via microbroth dilution methods.[21] Clinically, infections were also refractory to therapy, with longer infection duration observed among chronic granulomatous disease (CGD) patients with IA due to *A. udagawae* (mean duration 35 weeks) compared to CGD patients with IA due to *A. fumigatus* (mean duration 5.5 weeks).[21] High MICs of amphotericin B and voriconazole have also been observed in other reports of invasive infection due to *A. udagawae*.[6] In an ocular *A. udagawae* infection, therapy with itraconazole tablets and ophthalmic drops failed to clear the infection and the eye was exenterated. The isolate had a high MIC of voriconazole (8 μg/ml) but appeared to be susceptible to itraconazole based on a low MIC value (0.25 μg/ml).[23]

High MICs of itraconazole, voriconazole, and ravuconazole and susceptibility to amphotericin B and echinocandins were reported for two clinical isolates of *A. viridinutans*[11] that were later revealed to be *A. pseudofelis* (NRRL 62902) and *A. parafelis* (NRRL 62900).[4]

Other cases of human infection due to *A. udagawae* that were treated successfully with triazoles had high MIC values of nonazole antifungals. In one case of bronchial aspergillosis, decreased susceptibility to amphotericin B compared to *A. fumigatus sensu stricto* was observed.[22] In a patient with *A. udagawae* infection causing ARDS, treatment with amphotericin B and methylprednisone was unsuccessful, although no antifungal susceptibility testing was performed in this case.[75]

The extended spectrum triazole isavuconazole has been tested against one environmental and nine clinical isolates of *A. udagawae*.[92] Many of the isolates demonstrated decreased susceptibility to itraconazole (Mode MIC 1 μg/ml, geometric mean 0.660 μg/ml) and voriconazole (Mode MIC of 1 μg/ml, geometric mean 0.812 μg/ml) but increased susceptibility to isavuconazole (Mode MIC 0.125 μg/ml, geometric mean 0.100 μg/ml). This drug shows promise for clinical treatment of invasive aspergillosis due to *A. udagawae*. The susceptibility of other species in the AVC to isavuconazole is yet to be determined.

Antifungal susceptibilities of *A. felis* isolates from cats have been reported, with generally low MICs for amphotericin B and echinocandins. Some isolates had high MICs of triazoles, and cross-resistance to triazoles was also observed.[4,24] Results of antifungal susceptibility testing should inform antifungal selection in treatment of individual cats. Infecting isolates can be readily cultured from clinical biopsies of sinonasal mucosa or orbital masses on

Table 3. Antifungal susceptibility profiles reported for clinical isolates of *Aspergillus viridinutans* complex species and one environmental isolate (*A. viridinutans*) based on CLSI M38-A2,[4,11,21–23,26,27] EUCAST and Sensititre YO10 broth microdilution methods.[24]

Drug (ECV μg/ml for wild-type *A. fumigatus sensu stricto*)	Species	Number of isolates tested	MIC/MEC range (μg/ml)	MIC/MEC Geometric Mean (μg/ml)
Amphotericin B* (2)[89]	*A. felis*[4,24,26]	15	0.25–1	0.55
	A. parafelis[4,11,27]	5	0.37–2	0.99
	A. pseudofelis[4]	2	2	2.00
	A. pseudoviridinutans[4]	2	2	2.00
	A. udagawae[6,21–23]	14	0.125–4	1.53
	A. viridinutans[4]	1	0.25	0.25
Itraconazole* (0.25)[90]	*A. felis*[4,24,26]	15	0.03–8	0.31
	A. parafelis[4]	5	14.4–>16	15.17
	A. pseudofelis[4]	2	>16	>16.00
	A. pseudoviridinutans[4]	2	2–>16	5.65
	A. udagawae[6,21–23]	14	0.125–4	0.48
	A. viridinutans[4]	1	1	1.00
Voriconazole* (1)[90]	*A. felis*[4,24,26]	15	0.25–8	1.45
	A. parafelis[4]	5	4–8	5.28
	A. pseudofelis[4]	2	8	8.00
	A. pseudoviridinutans[4]	2	2–8	4.00
	A. udagawae[6,21–23]	14	0.25–>16	1.12
	A. viridinutans[4]	1	1	1.00
Posaconazole* (0.25)[90]	*A. felis*[24,26]	14	0.03–4	0.14
	A. parafelis[4]	3	0.25–0.41	0.29
	A. udagawae[21,23]	5	0.25–0.5	0.33
Terbinafine*	*A. felis*[24]	13	0.25	0.25
	A. parafelis[4]	2	0.75–1.2	0.95
	A. udagawae[21]	4	0.25–1	0.40
Caspofungin† (1)[91]	*A. felis*[24,26]	14	0.008–2	0.06
	A. parafelis[4]	3	<0.016–1	0.82
	A. udagawae[6,21,23]	12	0.015–0.5	0.05
Anidulafungin†	*A. felis*[24,26]	14	0.015–<0.03	0.02
	A. parafelis[4]	1	<0.016	<0.02
	A. udagawae[23]	1	0.016	0.02
Micafungin†	*A. felis*[24,26]	14	0.008–<0.03	0.01
	A. parafelis[4]	2	0.03–2	0.07
	A. udagawae[21–23]	6	<0.015–0.25	0.25
Fluconazole*	*A. udagawae*[23]	1	>256	>256.00

*MIC, minimum inhibitory concentration; †MEC, minimum effective concentration.

commercial media, for example, Sabouraud's dextrose agar or malt extract agar at 37°C.[71]

The *Aspergillus viridinutans* complex contains many clinically relevant species and recently discovered species of unknown pathogenicity. Known pathogenic species in the complex can affect both human and animal patients, reflecting the scope for a One-Health approach to disease investigation. Human and veterinary clinicians should be aware of the emerging threat members of this complex pose to susceptible patients. Knowledge of their epidemiology is important for exposure risk assessment and differential diagnoses. Their seemingly global distribution further highlights the need for clinician awareness. Members of this complex share some morphological and physiological characteristics and relatedness is confirmed by phylogeny based on combined multi-locus sequence analysis. Given that members of the *A. viridinutans* complex have seemingly inherent resistance to commonly used triazole antifungal therapeutics and infections usually have a poor to grave clinical prognosis, it is important to correctly identify agents of disease through polyphasic taxonomy and to perform antifungal susceptibility testing. This will assist both human and veterinary practitioners with prognosis assessment and case management.

Acknowledgments

The authors wish to thank Dr Jos Houbraken of the Westerdijk Fungal Biodiversity Institute for technical assistance with fungal light microscopy imaging.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this manuscript.

References

1. Frisvad JC, Larsen TO. Extrolites of *Aspergillus fumigatus* and other pathogenic species in *Aspergillus* section *Fumigati*. *Front Microbiol*. 2016; **6**: 1–14.
2. Barrs VR, Beatty JA, Dhand NK et al. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. *Vet J*. 2014; **201**: 215–222.
3. Howard SJ. Multi-resistant aspergillosis due to cryptic species. *Mycopathologia*. 2014; **178**: 435–439.
4. Sugui JA, Peterson SW, Figat A et al. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J Clin Microbiol*. 2014; **52**: 3707–3721.
5. Balajee SA, Gribskov JL, Hanley E et al. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. *Eukaryot Cell*. 2005; **4**: 625–632.
6. Balajee SA, Nickle D, Varga J et al. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryot Cell*. 2006; **5**: 1705–1712.
7. Zbinden A, Imhof A, Wilhelm MJ et al. Fatal outcome after heart transplantation caused by *Aspergillus lentulus*. *Transpl Infect Dis*. 2012; **14**: E60–E63.
8. Balajee SA, Kano R, Baddley JW et al. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *J Clin Microbiol*. 2009; **47**: 3138–3141.
9. Montenegro G, Sánchez Puch S, Jewtuchowicz VM et al. Phenotypic and genotypic characterization of *Aspergillus lentulus* and *Aspergillus fumigatus* isolates in a patient with probable invasive aspergillosis. *J Med Microbiol*. 2009; **58** (Pt 3): 391–395.
10. Alhambra A, Catalán M, Moragues MD et al. Isolation of *Aspergillus lentulus* in Spain from a critically ill patient with chronic obstructive pulmonary disease. *Rev Iberoam Micol*. 2008; **25**: 246–249.
11. Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A et al. *Aspergillus* Section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. *Antimicrob Agents Chemother*. 2008; **52**: 1244–1251.
12. Kidd SE, Goeman E, Meis JF et al. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses*. 2015; **58**: 350–355.
13. Sabino R, Veríssimo C, Parada H et al. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. *Med Mycol*. 2014; **52**: 519–529.
14. Escribano P, Peláez T, Muñoz P et al. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? *Antimicrob Agents Chemother*. 2013; **57**: 2815–2820.
15. Yoshida H, Seki M, Umeyama T et al. Invasive pulmonary aspergillosis due to *Aspergillus lentulus*: Successful treatment of a liver transplant patient. *J Infect Chemother*. 2015; **21**: 479–481.
16. Mortensen KL, Johansen HK, Fuursted K et al. A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. *Eur J Clin Microbiol Infect Dis*. 2011; **30**: 1355–1363.
17. Symoens F, Haase G, Pihet M et al. Unusual *Aspergillus* species in patients with cystic fibrosis. *Med Mycol*. 2010; **48** (S1): S10–S16.
18. de Azevedo Bastos VR, de Castro Lima Santos DW, Padovan ACB et al. Early invasive pulmonary aspergillosis in a kidney transplant recipient caused by *Aspergillus lentulus*: first Brazilian report. *Mycopathologia*. 2015; **179**: 299–305.
19. Negri CE, Gonçalves SS, Xafranski H et al. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. *J Clin Microbiol*. 2014; **52**: 3633–3640.
20. Balajee SA, Gribskov J, Brandt M et al. Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. *J Clin Microbiol*. 2005; **43**: 5996–5999.
21. Vinh DC, Shea YR, Sugui JA et al. Invasive aspergillosis due to *Neosartorya udagawae*. *Clin Infect Dis*. 2009; **49**: 102–111.
22. Gyotoku H, Izumikawa K, Ikeda H et al. A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. *Med Mycol*. 2012; **50**: 631–636.
23. Posteraro B, Mattei R, Trivella F et al. Uncommon *Neosartorya udagawae* fungus as a causative agent of severe corneal infection. *J Clin Microbiol*. 2011; **49**: 2357–2360.
24. Barrs VR, van Doorn TM, Houbraken J et al. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One*. 2013; **8**: e64871.
25. Alvarez-Pérez S, Mellado E, Serrano D et al. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. *J Med Microbiol*. 2014; **63** (Pt 4): 617–619.
26. Peláez T, Alvarez-Pérez S, Mellado E et al. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. *J Med Microbiol*. 2013; **62** (Pt 3): 474–478.
27. Coelho D, Silva S, Vale-Silva L et al. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. *Med Mycol*. 2011; **49**: 755–759.
28. Katz ME, Dougall AM, Weeks K et al. Multiple genetically distinct groups revealed among clinical isolates identified as atypical *Aspergillus fumigatus*. *J Clin Microbiol*. 2005; **43**: 551–555.
29. Vinh DC, Shea YR, Jones PA et al. Chronic invasive aspergillosis caused by *Aspergillus viridinutans*. *Emerg Infect Dis*. 2009; **15**: 1292–1294.
30. Shigeyasu C, Yamada M, Nakamura N et al. Keratomycosis caused by *Aspergillus viridinutans*: an *Aspergillus fumigatus*-resembling mold presenting distinct clinical and antifungal susceptibility patterns. *Med Mycol*. 2012; **50**: 525–528.
31. Nováková A, Hubka V, Dudová Z et al. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (USA) and revision of *A. viridinutans* complex. *Fungal Diversity*. 2014; **64**: 253–274.

32. Fennell DI, Raper KB. New species and varieties of *Aspergillus*. Mycologia. 1955; **47**: 71–75.
33. Raper KB, Fennell DI. *The Genus Aspergillus*. Baltimore: Williams and Wilkins; 1965.
34. Horie Y, Miyaji M, Nishimura K et al. New and interesting species of *Neosartorya* from Brazilian soil. *Mycoscience*. 1995; **36**: 199–204.
35. Matsuzawa T, Takaki GMC, Yaguchi T et al. *Aspergillus arcoverdensis*, a new species of *Aspergillus* section *Fumigati* isolated from caatinga soil in State of Pernambuco, Brazil. *Mycoscience*. 2015; **56**: 123–131.
36. Eamvijarn A, Manoch L, Chamswarng C et al. *Aspergillus siamensis* sp. nov. from soil in Thailand. *Mycoscience*. 2013; **54**: 401–405.
37. Samson RA, Hong S, Peterson SW et al. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Stud Mycol*. 2007; **59**: 147–203.
38. Samson RA, Visagie CM, Houbraken J et al. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud Mycol*. 2014; **78**: 141–173.
39. McLennan EI, Ducker SC, Thrower LB. New soil fungi from Australian heathland: *Aspergillus, Penicillium, Spegazzinia*. *Aust J Bot*. 1954; **2**: 355–364.
40. Subramanian CV. The perfect states of *Aspergillus*. *Curr Sci India*. 1972; **41**: 755–761.
41. Malloch D, Cain RF. The *Trichocomataceae*: Ascomycetes with *Aspergillus, Paecilomyces* and *Penicillium* imperfect states. *Can J Bot*. 1972; **50**: 2613–2628.
42. Samson RA, WG. *Aspergillus aureoluteus*. In: Samson RA, Pitt JI, eds. *Advances in Penicillium and Aspergillus Systematics*. New York: Springer Science+Business Media, 1985, 31–54.
43. Hong SB, Go SJ, Shin HD et al. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia*. 2005; **97**: 1316–1329.
44. McNeill J, Barrie FR, Buck WR et al. International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) adopted by the Eighteenth International Botanical Congress Melbourne, Australia, July 2011. *Königstein: Koeltz Scientific Books*. 2012.
45. White TJ, Bruns T, Lee SJWT. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York: Academic Press, 1990, 315–322.
46. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol*. 1995; **61**: 1323–1330.
47. Schmitt I, Crespo A, Divakar PK et al. New primers for promising single-copy genes in fungal phylogenetics and systematics. *Persoonia*. 2009; **23**: 35–40.
48. Liu YJ, Whelen S, Hall BD. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Mol Biol Evol*. 1999; **16**: 1799–1808.
49. Peterson SW. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia*. 2008; **100**: 205–226.
50. Lillehoj EB, Milburn MS. Viriditoxin production by *Aspergillus viridi-nutans* and related species. *Appl Microbiol*. 1973; **26**: 202–205.
51. Lillehoj EB, Ciegler A. A toxic substance from *Aspergillus viridinutans*. *Can J Microbiol*. 1972; **18**: 193–197.
52. Hong SB, Shin HD, Hong J et al. New taxa of *Neosartorya* and *Aspergillus* in *Aspergillus* section *Fumigati*. *Antonie Van Leeuwenhoek*. 2008; **93**: 87–98.
53. Weisleder D, Lillehoj EB. Structure of viriditoxin, a toxic metabolite of *Aspergillus viridinutans*. *Tetrahedron Lett*. 1971; **48**: 4705–4706.
54. Cole RJ, Cox RH. *Handbook of Toxic Fungal Metabolites*. New York: Academic Press; 1981.
55. Gonçalves VN, Cantrell CL, Wedge DE et al. Fungi associated with rocks of the Atacama Desert: taxonomy, distribution, diversity, ecology and bioprospection for bioactive compounds. *Environ Microbiol*. 2016; **18**: 232–245.
56. Ramos AA, Prata-Sena M, Castro-Carvalho B et al. Potential of four marine-derived fungi extracts as anti-proliferative and cell death-inducing agents in seven human cancer cell lines. *Asian Pac J Trop Med*. 2015; **8**: 798–806.
57. Buttachon S, Chandrapatya A, Manoch L et al. Sartorymensin, a new indole alkaloid, and new analogues of tryptoquivaline and fiscalins produced by *Neosartorya siamensis* (KUFC 6349). *Tetrahedron*. 2012; **68**: 3253–3262.
58. Mendes G, Gonçalves VN, Souza-Fagundes EM et al. Antifungal activity of extracts from Atacama Desert fungi against *Paracoccidioides brasiliensis* and identification of *Aspergillus felis* as a promising source of natural bioactive compounds. *Mem Inst Oswaldo Cruz*. 2016; **111**: 209–217.
59. Xiao L, Liu H, Wu N et al. Characterization of the high cytochalasin E and rosellichalasin producing-*Aspergillus* sp. nov. F1 isolated from marine solar saltern in China. *World J Microbiol Biotechnol*. 2013; **29**: 11–17.
60. Naruse N, Yamamoto H, Murata S et al. Aspochalasin E, a new antibiotic isolated from a fungus. *J Antibiot (Tokyo)*. 1993; **46**: 679–681.
61. Tamiya H, Ochiai E, Kikuchi K et al. Secondary metabolite profiles and antifungal drug susceptibility of *Aspergillus fumigatus* and closely related species, *Aspergillus lentulus, Aspergillus udagawae*, and *Aspergillus viridinutans*. *J Infect Chemother*. 2015; **21**: 385–391.
62. National Center for Biotechnology Information. Basic Local Alignment Search Tool (BLAST): Standard Nucleotide BLAST. https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed 2016.
63. Verwer PEB, van Leeuwen WB, Girard V et al. Discrimination of *Aspergillus lentulus* from *Aspergillus fumigatus* by Raman spectroscopy and MALDI-TOF MS. *Eur J Clin Microbiol Infect Dis*. 2014; **33**: 245–251.
64. Alastruey-Izquierdo A, Mellado E, Peláez T et al. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrob Agents Chemother*. 2013; **57**: 3380–3387.
65. Gautier M, Normand AC, Ranque S. Previously unknown species of *Aspergillus*. *Clin Microbiol Infect*. 2016; **S1198-743X**: 30140–30149.
66. Varga J, Tóth B, Rigó K et al. Genetic variability within the *Aspergillus viridinutans* species. *Folia Microbiol (Praha)*. 2000; **45**: 423–428.
67. Staab JF, Balajee SA, Marr KA. *Aspergillus* section *Fumigati* typing by PCR-restriction fragment polymorphism. *J Clin Microbiol*. 2009; **47**: 2079–2083.

68. Adhikari M, Kim S, Yadav DR et al. A new record of *Neosartorya aureola* isolated from field soil in Korea. *Korean J Mycol*. 2015; **43**: 191–195.
69. Talbot JJ, Johnson LR, Martin P et al. What causes canine sinonasal aspergillosis? A molecular approach to species identification. *Vet J*. 2014; **200**: 17–21.
70. Barrs VR, Talbot JJ. Feline aspergillosis. *Vet Clin North Am Small Anim Pract*. 2014; **44**: 51–73.
71. Barrs VR, Halliday C, Martin P et al. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *Vet J*. 2012; **191**: 58–64.
72. Kano R, Itamoto K, Okuda M et al. Isolation of *Aspergillus udagawae* from a fatal case of feline orbital aspergillosis. *Mycoses*. 2008; **51**: 360–361.
73. Kano R, Shibahashi A, Fujino Y et al. Two cases of feline orbital aspergillosis due to *Aspergillus udagawae* and *A. viridinutans*. *J Vet Med Sci*. 2013; **75**: 7–10.
74. Tamborini A, Robertson E, Talbot JJ et al. Sinonasal aspergillosis in a British Shorthair cat in the UK. *J Feline Med Surg Open Reports*. 2016; **2**: 1–6.
75. Farrell JJ, Kasper DJ, Taneja D et al. Acute respiratory distress caused by *Neosartorya udagawae*. *Med Mycol Case Rep*. 2014; **24**: 1–5.
76. Sugui JA, Vinh DC, Nardone G et al. *Neosartorya udagawae* (*Aspergillus udagawae*), an emerging agent of aspergillosis: how different is it from *Aspergillus fumigatus*? *J Clin Microbiol*. 2010; **48**: 220–228.
77. Andersen CT, Bechensteen AG, Hesstvedt L et al. Multiple brain abscesses caused by *Aspergillus viridinutans* in a leukemic child. *Mycoses*. 2012; **55** (S4): 75.
78. Gonçalves SM, Lagrou K, Duarte-Oliveira C et al. The microbiome-metabolome crosstalk in the pathogenesis of respiratory fungal diseases. *Virulence*. 2016; Nov 7 epub ahead of print http://dx.doi.org/10.1080/21505594.2016.1257458.
79. Patterson TF, Thompson GR, III, Denning DW et al. Practical guidelines for the diagnosis and management of aspergillosis: 2016 update by the infectious diseases society of America. *Clin Infect Dis*. 2016; **63**: e1–e60.
80. Quimby JM, Hoffman SB, Duke J et al. Adverse neurologic events associated with voriconazole use in 3 cats. *J Vet Intern Med*. 2010; **24**: 647–649.
81. Smith LN, Hoffman SB. A case series of unilateral orbital aspergillosis in three cats and treatment with voriconazole. *Vet Opthalmol*. 2010; **13**: 190–203.
82. van der Linden JW, Warris A, Verweij PE. *Aspergillus* species intrinsically resistant to antifungal agents. *Med Mycol*. 2011; **49** (S1): S82–S89.
83. Howard SJ, Arendrup MC. Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection. *Med Mycol*. 2011; **49** (S1): S90–S95.
84. Chen J, Li H, Li R et al. Mutations in the cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *J Antimicrob Chemother* 2005; **55**: 31–37.
85. Verweij PE, Kema GH, Zwaan B et al. Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould *Aspergillus fumigatus*. *Pest Manag Sci*. 2013; **69**: 165–170.
86. Kusuya Y, Takahashi-Nakaguchi A, Takahashi H et al. Draft genome sequence of the pathogenic filamentous fungus *Aspergillus udagawae* strain IFM 46973T. *Genome Announc*. 2015; **3**: e00834–15.
87. Mellado E, Alcazar-Fuoli L, Cuenca-Estrella M et al. Role of *Aspergillus lentulus* 14-α sterol demethylase (Cyp51A) in azole drug susceptibility. *Antimicrob Agents Chemother*. 2011; **55**: 5459–5468.
88. Alcazar-Fuoli L, Cuesta I, Rodriguez-Tudela JL et al. Three-dimensional models of 14$\alpha\alpha$-sterol demethylase (Cyp51A) from *Aspergillus lentulus* and *Aspergillus fumigatus*: an insight into differences in voriconazole interaction. *Int J Antimicrob Agents*. 2011; **38**: 426–434.
89. Espinel-Ingroff A, Cuenca-Estrella M, Fothergill A et al. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Antimicrob Agents Chemother*. 2011; **55**: 5150–5154.
90. Espinel-Ingroff A, Diekema DJ, Fothergill A et al. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 Document). *J Clin Microbiol*. 2010; **48**: 3251–3257.
91. Espinel-Ingroff A, Fothergill A, Fuller J et al. Wild-type MIC distributions and epidemiological cutoff values for caspofungin and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 Document). *Antimicrob Agents Chemother*. 2011; **55**: 2855–2859.
92. Datta K, Rhee P, Byrnes E, III et al. Isavuconazole activity against *Aspergillus lentulus*, *Neosartorya udagawae*, and *Cryptococcus gattii*, emerging fungal pathogens with reduced azole susceptibility. *J Clin Microbiol*. 2013; **51**: 3090–3093.
93. Hong SB, Kim DH, Park IC et al. Re-identification of *Aspergillus fumigatus* sensu lato based on a new concept of species delimitation. *J Microbiol*. 2010; **48**: 607–615.
94. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol*. 1980; **16**: 111–120.
95. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985; **39**: 783–791.
96. Tamura K, Stecher G, Peterson D et al. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*. 2013; **30**: 2725–2729.

Appendix 2: Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for human and animal fungal pathogens

PLOS ONE

RESEARCH ARTICLE

Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for animal and human fungal pathogens

Jessica J. Talbot[1], Jos Houbraken[2], Jens C. Frisvad[3], Robert A. Samson[2], Sarah E. Kidd[4], John Pitt[5], Sue Lindsay[6], Julia A. Beatty[1], Vanessa R. Barrs[1]*

1 Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camperdown, New South Wales, Australia, **2** Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands, **3** Department of Biotechnology and Biomedicine, Technical University of Denmark, Kongens Lyngby, Denmark, **4** National Mycology Reference Centre, Microbiology and Infectious Diseases, SA Pathology, Adelaide, South Australia, Australia, **5** CSIRO Food Science, CSIRO, North Ryde, New South Wales, Australia, **6** Faculty of Science and Engineering, Macquarie University, North Ryde, New South Wales, Australia

* vanessa.barrs@sydney.edu.au

OPEN ACCESS

Citation: Talbot JJ, Houbraken J, Frisvad JC, Samson RA, Kidd SE, Pitt J, et al. (2017) Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for animal and human fungal pathogens. PLoS ONE 12(8): e0181660. https://doi.org/10.1371/journal.pone.0181660

Editor: Olaf Kniemeyer, Leibniz-Institut fur Naturstoff-Forschung und Infektionsbiologie eV Hans-Knoll-Institut, GERMANY

Received: March 31, 2017

Accepted: July 5, 2017

Published: August 9, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was funded by an Australian Endeavour Research Fellowship (JT), a Thomson Fellowship (VB) and a University of Sydney Compacts Grant (VB). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Invasive fungal infections (IFI) due to species in *Aspergillus* section *Fumigati* (ASF), including the *Aspergillus viridinutans* species complex (AVSC), are increasingly reported in humans and cats. The risk of exposure to these medically important fungi in Australia is unknown. Air and soil was sampled from the domiciles of pet cats diagnosed with these IFI and from a nature reserve in Frankston, Victoria, where *Aspergillus viridinutans sensu stricto* was discovered in 1954. Of 104 ASF species isolated, 61% were *A. fumigatus sensu stricto*, 9% were AVSC (*A. felis*-clade and *A. frankstonensis* sp. nov.) and 30% were other species (30%). Seven pathogenic ASF species known to cause disease in humans and animals (*A. felis*-clade, *A. fischeri*, *A. thermomutatus*, *A. lentulus*, *A. laciniosus A. fumisynnematus*, *A. hiratsukae*) comprised 25% of isolates overall. AVSC species were only isolated from Frankston soil where they were abundant, suggesting a particular ecological niche. Phylogenetic, morphological and metabolomic analyses of these isolates identified a new species, *A. frankstonensis* that is phylogenetically distinct from other AVSC species, heterothallic and produces a unique array of extrolites, including the UV spectrum characterized compounds DOLD, RAIMO and CALBO. Shared morphological and physiological characteristics with other AVSC species include slow sporulation, optimal growth at 37˚C, no growth at 50˚C, and viriditoxin production. Overall, the risk of environmental exposure to pathogenic species in ASF in Australia appears to be high, but there was no evidence of direct environmental exposure to AVSC species in areas where humans and cats cohabitate.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Aspergillosis is an opportunistic respiratory or systemic disease affecting a range of mammalian, avian and reptile hosts globally. It is most commonly caused by fungi belonging to *Aspergillus* section *Fumigati* (ASF), of which 19 of 63 described species are known to be pathogenic [1, 2]. The saprophytic, ubiquitous fungus *Aspergillus fumigatus sensu stricto* is the most common cause of aspergillosis overall. However, other species within the section, including members of the *Aspergillus viridinutans* species complex (AVSC) are increasingly recognized as emerging causes of invasive fungal infections (IFI). Currently, of the 10 accepted species in the AVSC, six are known pathogens including *A. udagawae*, *A. felis*, *A. wyomingensis*, *A. pseudoviridinutans, A. parafelis and A. pseudofelis* [2–8].

Aspergillus felis, *A. wyomingensis* and *A. udagawae* cause an invasive form of fungal rhinosinusitis in systemically immunocompetent cats called sino-orbital aspergillosis [2, 4]. Infection is often fatal, with high MICs of azole antifungals recorded *in vitro* and clinical resistance *in vivo* [4, 9]. *Aspergillus felis* has been isolated from clinical samples in humans, cats, dogs and birds, demonstrating a broad range of susceptible hosts [4]. While feline sino-orbital aspergillosis has been reported in diverse geographic regions including Europe, the United States and Japan, the majority of cases are from Australia [4, 10]. Other, less common causes of feline fungal rhinosinusitis include *A. fumigatus s. str.*, *A. thermomutatus* (syn. *N. pseudofischeri*), *A. fischeri*, *A. lentulus* and *A. niger* species complex [9–13].

Aspergillus felis was first isolated from infected cats in Australia [4]. It has since been isolated from environmental samples from the USA and from other countries with low to no disease incidence, in soil (Czech Republic, India, Zambia) [14], Caribbean Pine (Sri Lanka) [14], indoor air (Germany) [4] and on desert rocks in Chile [15]. The environmental prevalence of *A. felis* and other pathogenic species in section *Fumigati* in Australia has not been investigated, so environmental risk factors for exposure are unknown.

Here we report the findings of an investigation to determine if ASF species causing IFI in cats could be isolated from the home environments of cats with these infections. We also collected environmental samples from the region where *A. viridinutans s. str.* was first recovered and discovered a novel species, *Aspergillus frankstonensis* sp. nov.

Materials and methods

Sampling and isolation

With the permission of private land owners, as per the University of Sydney Human Ethics Approval (project number 2014/980), soil and air samples were collected from yards and gardens where eight cats previously diagnosed with SOA due to *A. udagawae* (n = 2), *A. felis* (n = 5) and *A. wyomingensis* (n = 1) were domiciled (5 in New South Wales (NSW), 2 in Victoria (VIC), 1 in the Australian Capital Territory (ACT). These locations included urban (n = 6), rural (n = 1) and semi-rural (n = 1) properties. Samples were also collected from municipal nature reserves in the coastal town of Frankston in the Mornington Peninsula region, VIC, where *A. viridinutans s. str.* was originally isolated in soil and rabbit dung [16]. Permission to sample was granted by the Frankston City Council. Field studies did not involve endangered or protected species.

A minimum of two air samples were collected at each site directly onto dichloran rose-bengal agar (DRBC) (Thermo Scientific, Thebarton, Australia) using a Merck Millipore MAS-100 NT® air sampler (Merck KGaA, Darmstadt, Germany) at a rate of 100L/minute [17]. Ten soil samples, including garden and lawn soils (sandy and loamy) and mulches (sugar cane, wood chip and straw hay) were collected at each site to a depth of 10 cm and stored at 4˚C. Isolation

of fungi from soil was achieved by 10^{-1} to 10^{-4} serial dilutions with sterile water. The resulting dilutions were transferred to a Whirlpak® (Nasco) homogenizer bag, heated at 75˚C for 30 min, and 0.1 mL was inoculated onto DRBC [14, 18]. Additionally, 17 representative samples of different soil types from each location were processed using the same method without heat treatment.

Samples of commercial cat litter substrates, including the same brands used by cats in the study (recycled paper pellets, clumping clay, recycled timber pellets and silica crystals), were processed as for soil samples and using direct plating. Stored and unopened commercially available dry cat food samples were also tested in the same manner.

Air samples were incubated in the dark for up to three weeks at 37˚C. Treated soil samples were cultured on DRBC and malt extract agar supplemented with chloramphenicol (MEASC) [19] for up to three weeks at 37˚C. Fungal colonies were sub-cultured onto malt extract agar (MEA) (Thermo Scientific Oxoid Microbiology Products, Thebarton, Australia; Landsmeer, Netherlands) [19] for further analysis.

Identification of environmental isolates

For environmental isolates with gross macro- and microscopic morphological features consistent with *Aspergillus* spp. (excluding *Aspergillus* section *Nigri* species), amplification and sequencing of the ITS region and partial β-tubulin (*BenA*) gene was performed for species identification as previously described [20, 21]. A BLAST [22] search on GenBank was performed with the newly generated sequences, which were also aligned with *Aspergillus* references sequences (Samson et al. 2014) using *MEGA* version 6 software [23]. Phylogenetic analysis was performed using the maximum likelihood discrete method (tree searching method of 1000 replicate trees) and bootstrapping to determine the statistical support of the nodes.

Phylogenetic analysis of AVSC isolates. Nine environmental isolates identified in the AVSC were further examined based on molecular studies and phylogenetic analysis results of less than 100% match for *A. felis* (n = 7) and *A. viridinutans* (n = 2) ITS and *BenA* sequences. Additional molecular analysis was performed by sequencing partial calmodulin (*CaM*), actin (*Act*) and RNA polymerase II second largest subunit (*RPB2*) genes as previously described [8, 24]. Specific primers targeting the minichromosome maintenance factor gene (*Mcm7*) were developed based on previously published primers [8]: MCM7-709F_Fum ACTCGTGTCTCG GACGTCAAACC (forward) and MCM7-1348R_Fum GATTTGGCRACACCAGGATCACCCAT (reverse). For comparative analysis, these genes were also sequenced for AVSC members in the CBS-KNAW collection and for a new clinical isolate from the USA from a cat with SOA (see Table 1). Phylogenetic and molecular evolutionary analyses were conducted using Randomised Axelerated Maximum Likelihood (RAxML) and Bayesian methods [25, 26].

Phenotypic species differentiation. The physiology and macro- and micromorphology of the two isolates demonstrated to be phylogenetically distinct from other AVSC species (CBS 142234 and CBS 142233) were studied. Isolates were grown at 25˚C on Czapek yeast agar (CYA) [19], Czapek yeast agar with 5% NaCl (CYAS) [27], yeast extract sucrose agar (YES) [19], MEA, oatmeal agar (OAT), creatine sucrose agar (CREA) [19], dichloran 18% glycerol agar (DG18) [28] for seven days. For temperature growth testing isolates were also grown on CYA at 30˚C, 37˚C, 45˚C and 50˚C for seven days.

Extrolite analysis. Extrolite extraction was performed on the two phylogenetically distinct isolates after growth on CYA and YES agar at 25˚C and 37˚C for 7 days. Three agar plugs were extracted according to the agar plug extraction method of Smedsgaard [29]. Extracts were analysed using UHPLC-DAD (Dionex Ultramate 3000 UHPLC) and compounds were identified

Table 1. Isolates included in phylogenetic analysis of the *Aspergillus viridinutans* complex.

Identification number	Species name	Source	Location	ITS	BenA	Cam	RPB2	Actin	MCM7
DTO 006-A3	*A. udagawae*	soil	USA	KY808735	KY808572	KY808696	KY808908	KY808509	KY808858
DTO 019-D7	*A. udagawae*	unknown	unknown	KY808737	KY808573	KY808697	KY808909	KY808510	KY808859
DTO 019-D8	*A. udagawae*	unknown	unknown	KY808738	KY808574	KY808698	KY808910	KY808511	KY808860
DTO 019-F2	*A. arcoverdensis*	soil	Australia	KY808747	KY808575	KY808699	KY808911	KY808512	KY808861
DTO 050-F1, CBS 127.56^{T}, NRRL 4365	*A. viridinutans*	rabbit dung	Australia	EF669978	AF134779	DQ534162	EF669765	DQ094862	KY808862
DTO 331-G6, CBS 105.55^{T}, DTO 052-C8, NRRL 2244	*A. aureolus*	soil	Ghana	EF669950	EF669808	KY808720	KY808943	DQ094861	KY808895
DTO 131-E3	*A. felis*	cat, RBM	Australia	JX021671	KY808576	KY808701	KY808912	KY808513	KY808863
DTO 131-E4	*A. felis*	cat, RBM	Australia	JX021673	JX021692	KY808702	KY808913	KY808514	KY808864
DTO 131-E6, CBS 130244	*A. felis*	cat, RBM	Australia	JX021675	JX021694	JX021717	KY808915	KY808516	KY808866
DTO 131-E5	*A. felis*	cat, RBM	Australia	JX021674	JX021693	JX021719	KY808914	KY808515	KY808865
DTO 131-E9	*A. felis*	cat, RBM	Australia	JX021676	JX021696	KY808703	KY808916	KY808517	KY808867
DTO 131-F1	*A. felis*	cat, RBM	Australia	JX021677	JX021697	KY808704	KY808917	KY808518	KY808868
DTO 131-F2	*A. felis*	cat, RBM	Australia	JX021678	JX021698	KY808705	KY808918	KY808519	KY808869
DTO 131-F3	*A. felis*	cat, RBM	Australia	JX021679	JX021699	KY808706	KY808919	KY808520	KY808870
DTO 131-F4, CBS 130245^{T}	*A. felis*	cat, RBM	Australia	J X021685	JX021700	JX021715	KY808920	KY808521	KY808871
DTO 131-F6	*A. felis*	cat, RBM	Australia	JX021680	JX021702	JX021721	KY808921	KY808522	KY808872
DTO 131-F9, CBS 130246	*A. felis*	cat, SNC	Australia	JX021681	JX021704	JX021724	KY808922	KY808523	KY808873
DTO 131-G1	*A. felis*	cat, RBM	Australia	JX021682	JX021705	JX021725	KY808923	KY808524	KY808874
DTO 131-G2, CBS 130247	*A. felis*	cat, RBM	Australia	JX021683	JX021706	JX021726	KY808924	KY808525	KY808875
DTO 131-G3, CBS 130248	*A. felis*	cat, RBM	Australia	JX021684	JX021707	JX021727	KY808925	KY808526	KY808876
DTO 155-G2	*A. wyomingensis*	cat, RBM	Australia	JX021685	JX021709	KY808707	KY808927	KY808527	KY808878
DTO 155-G3, CBS 130249	*A. felis*	dog, VH	Australia	JX021686	JX021711	JX021713	KY808928	KY808528	KY808879
DTO 157-D7, CBS 114217^{T}	*A. udagawae*	soil	Brazil	AB250781	AF132226	AB748566	KY808929	KY808529	KY808880
DTO 157-D8, CBS 114218	*A. udagawae*	soil	Brazil	AB250782	AB248303	AY689373	KY808930	KY808530	KY808881
DTO 159-C9, CBS 130250	*A. felis*-clade	cat, RBM	United Kingdom	JX021689	JX021712	JX021714	KY808931	KY808531	KY808882
DTO 166-D6	*A. udagawae*	cat	Australia	KY808740	KY808579	KY808710	KY808932	KY808532	KY808883
DTO 175-H3	*A. sp.*	surface water	Portugal	KY808741	KY808580	KY808711	KY808933	KY808533	KY808884
DTO 176-F1	*A. felis*	air	Germany	KY808742	KY808581	KC305168	KY808934	KY808534	KY808885
DTO 278-B6, CBS 137452^{T}	*A. siamensis*	soil	Thailand	-	KY808582	AB776704	KY808712	KY829134	KY808886
DTO 278-B7, CBS 137453	*A. aureolus*	soil	Brazil	KY808743	KY808583	KY808713	KY808935	KY808535	KY808887
DTO 283-D3	*A. udagawae*	soil	Thailand	KY808744	KY808584	KY808714	KY808936	KY808536	KY808888
DTO 303-A1	*A. pseudoviridinutans*	*Pinus caribea* (pine tree)	Sri Lanka	KY808745	KY808585	KY808715	KY808937	KY808537	KY808889
DTO 308-H6	*A. udagawae*	soil	Turkey	KY808746	KY808586	KY808716	KY808938	KY808539	KY808890
DTO 316-C8	*A. felis*/ *A. conversis*	CBS culture contaminant	The Netherlands	KY808750	KY808587	KY808717	KY808939	KY808540	KY808891
DTO 316-F7, CBS 139187^{T}	*A. arcoverdensis*	semi-desert soil	Brazil	KY808748	AB818845	AB818856	KY808940	KY808541	KY808892
DTO 316-F9, CBS 139188	*A. arcoverdensis*	unknown	Brazil	KY808749	KY808588	KY808718	KY808941	KY808542	KY808893
DTO 327-G4	*A. viridinutans*	human patient	The Netherlands	KY808751	KX903288	KY808719	KY808942	KY808543	KY808894
DTO 332-B1, CBS 135456^{T}	*A. wyomingensis*	coal mine reclamation site soil	Glenrock, USA	HG324081	KY808589	KY808721	HF937378	KY808544	KY808896
NRRL 6106, DTO 342-I3, CBS 140764	*A. pseudoviridinutans*	unknown	Unknown	-	AF134778	KJ914709	KY808965	KY808566	KJ914726
NRRL 62900, CM-3147, DTO 342-I4, CBS 140762^{T}	*A. felis*-clade	human, OPE	Spain	-	KJ914692	KJ914702	KY808966	KY808567	KJ914720
NRRL 62901, CM-5623, DTO 342-I5, CBS 140765	*A. felis*-clade	human, lungs	Portugal	-	KJ914693	KJ914703	KY808967	KY808568	KJ914721
NRRL 62902, CM-4518, DTO 342-I6, CBS 140766	*A. felis*-clade	human, nail	Spain	-	KJ914696	KJ914704	KY808968	KY808569	KJ914722
NRRL 62903, CM-6087, DTO 342-I7, CBS 140763^{T}	*A. felis*-clade	human, sputum	Spain	-	KJ914697	KJ914705	KY808969	KY808570	KJ914723

(*Continued*)

Table 1. (*Continued*)

Identification number	Species name	Source	Location	ITS	BenA	Cam	RPB2	Actin	MCM7
NRRL 62904, NIHAV1, DTO 304-I5, CBS 140396^T	*A. pseudoviridinutans*	human, lung	USA	-	GQ144441	GQ144442	KJ914730	KY808538	KJ914727
DTO 341-E8	*A. felis*-clade	woodland soil	Frankston, Australia	KY808757	KY808595	KY808725	KY808949	KY808550	KY808902
DTO 341-E9	*A. felis*-clade	woodland soil	Frankston, Australia	KY808758	KY808596	KY808726	KY808950	KY808551	KY808903
DTO 341-F1	*A. felis*-clade	woodland soil	Frankston, Australia	KY808759	KY808597	KY808727	KY808951	KY808552	KY808904
DTO 341-E6	*A. felis*-clade	woodland soil	Frankston, Australia	KY808755	KY808593	KY829133	KY808947	KY808548	KY808900
DTO341-E4	*A. felis*-clade	woodland soil	Frankston, Australia	KY808753	KY808591	KY808723	KY808945	KY808546	KY808898
DTO 341-F2	*A. felis*-clade	woodland soil	Frankston, Australia	KY808760	KY808598	KY808728	KY808952	KY808553	KY808905
DTO 341-E5	*A. felis*-clade	woodland soil	Frankston, Australia	KY808754	KY808592	KY829132	KY808946	KY808547	KY808899
OHIG B6-A1	*A. felis*-clade	cat, RBM	Connecticut, USA	KY808857	KY808695	KY808734	KY808970	KY808571	KY808907
DTO 341-E7, CBS 142233, IBT 34172	*A. frankstonensis* sp. nov.	woodland soil	Frankston, Australia	KY808756	KY808594	KY808724	KY808948	KY808549	KY808901
DTO 341-F3, CBS 142234, IBT 34204^T	*A. frankstonensis* sp. nov.	woodland soil	Frankston, Australia	KY808761	KY808599	KY808729	KY808953	KY808554	KY808906
DTO 341-E3, CBS 142231	*A. udagawae*	cat, RBM	Kealba, Australia	KY808752	KY808590	KY808722	KY808944	KY808545	KY808897
DTO 153-A1*, CBS 458.75	*A.* sp.	soil	India	KY808736	AY685178	HG426048	KY808926	DQ094853	KY808877

^{T} = Type strain; CBS ID number culture collection of the Westerdijk Fungal Biodiversity Institute, the Netherlands; DTO in-house collection ID number at Westerdijk Institute, the Netherlands; NRRL ID number Agricultural Research Service Culture Collection, USA; OHIG ID number One Health Infectious Disease Research Group Collection, University of Sydney, Australia; RBM = retrobulbar mass; SNC = sino-nasal cavity; VH = vitreous humor; OPE = oropharyngeal exudates.

* = This isolate was included in this study as previous in-house sequence analysis showed close phylogenetic relatedness to members of this complex.— = no sequence or accession number available.

https://doi.org/10.1371/journal.pone.0181660.t001

against an internal database of UV spectra and literature [30]. Extrolite standards were available as reported by Nielsen et al. [30].

Antifungal susceptibility testing. Antifungal susceptibility testing was performed on all AVSC environmental isolates, and the clinical isolate, using Sensititre YeastOne YO8 microdilution trays (Trek Diagnostic Systems, Thermo Fisher Scientific, Scoresby, Australia) to assess the minimum inhibitory concentration (MIC) values of posaconazole (POS), itraconazole (ITZ), voriconazole (VCZ), fluconazole (FLU), ketoconazole (KCZ), amphotericin B (AMB), and minimum effective concentration (MEC) of caspofungin (CSP) as previously described [31].

Mating type analysis. Mating type for all AVSC environmental isolates was determined by targeting the MAT1-1 and MAT1-2 genes [4]. Mating experiments were also performed on isolates with opposite mating types of the same species where available, or with other members of the AVSC where unavailable, on OAT and MEA in the dark at 30˚C. Ascospore viability tests were performed by rupturing ascomata, suspending ascospores in 0.05% Tween 80 and heating at 70˚C for 60 min. After heating, 100 μL of the ascospore suspension was plated on 2% MEA and incubated at 28˚C for 24 h [32]. To act as a negative control, the same treatment was also applied to the conidia of paired parental strains from the mating plate. Scanning electron microscopy was performed on all ascospores (Emitech 550K Sputter coater JEOL 6480LA).

Nomenclature. The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: PubMed Central, LOCKSS.

Results

Sequence-based identification

Overall 104 ASF species were isolated from all sites including 61% (n = 64) *A. fumigatus s. str.*, 9% (n = 9) AVSC (*A. felis*-clade and *A. frankstonensis* sp. nov.) and 30% (n = 31) other ASF species (see S2 Table, S1 Fig) (Genbank accession numbers: KY808753-KY808856 (ITS); KY808591- KY808694 (*BenA*)). Pathogenic ASF species (*A. A. felis*-clade (n = 7), *A. fischeri* (n = 6), *A. thermomutatus* (n = 1), *A. lentulus* (n = 2), *A. laciniosus* (n = 2), *A. fumisynnematus* (n = 4), *A. hiratsukae* (n = 1) comprised 25% of isolates overall. AVSC species were only isolated from Frankston soil where they were abundant and comprised 41% of isolates from that site.

Aspergillus isolates were only recovered from three of the nine sampled locations when no heat treatment was used. Isolated species included *A. fumigatus s. str.* (Kealba, n = 1), *A. hiratsukae* (Frankston, n = 1; Amaroo, n = 1). *Aspergillus fumigatus s. str.* was also isolated from recycled paper cat litter. No *Aspergillus* species were isolated from other cat litter types and dry food tested. The clinical isolate from the cat in the US (Table 1) was phylogenetically closely related to the type of *A. parafelis*, CBS 140762, in the *A. felis*-clade.

Phylogeny. The length of the datasets were *BenA* 422 basepairs (bp), *CaM* 485 bp, *Act* 379 bp, *RPB2* 831 bp and *Mcm7* 450 bp. Phylogenetic analysis of combined *BenA*, *CaM*, *Act*, *RPB2* and *Mcm7* data (2567 bp) confirmed seven isolates belong in the *A. felis*-clade, related to the type of *A. parafelis* CBS 140762, and two isolates were most closely related to, but phylogenetically distinct from *A. viridinutans s. str.* (Figs 1 and 2). These two isolates had identical sequences and are described as a new species below, *A. frankstonensis* sp. nov.

The individual and combined phylogenies (*BenA*, *CaM*, *RPB2*, *Act*, *Mcm7*) show that the 9 isolates from Frankston soil are accommodated in the AVSC and ASF (Figs 1 and 2). Two of these isolates (CBS 142233, CBS 142234) were most closely related to *A. viridinutans s. str.* in all trees, with bootstrap support (bs) >70% in four of the six trees generated (*RPB2* 71% bs; *Act* 95% bs; *BenA* 74% bs; combined 100% bs). This was also supported by Bayesian method posterior probability values (*RPB2* 0.99 pp; *Act* 1.00 pp; *BenA* 0.97 pp; combined 1.00 pp). *Aspergillus arcoverdensis* takes a basal position to those two species with high statistical support (bs 100%, 1.00 pp). These sequences of CBS 142234 and CBS 142233 are different from the other species in AVSC and ASF, with the genetic change seen in the distance of the horizontal branch. For both these isolates the percentage difference at ITS, *BenA*, *CaM*, *RPB2*, *Act* and *Mcm7* were 99%, 96%, 98%, 99%, 98% and 98% respectively when compared with *A. viridinutans s. str.* (NRRL 4365). A BLAST analysis did not show a 100% similarity match on GenBank for either of these isolates, and the highest similarities were with the type strain for *A. viridinutans s. str.* The remaining seven Frankston soil isolates (DTO 341-F2, DTO 341-E6, DTO

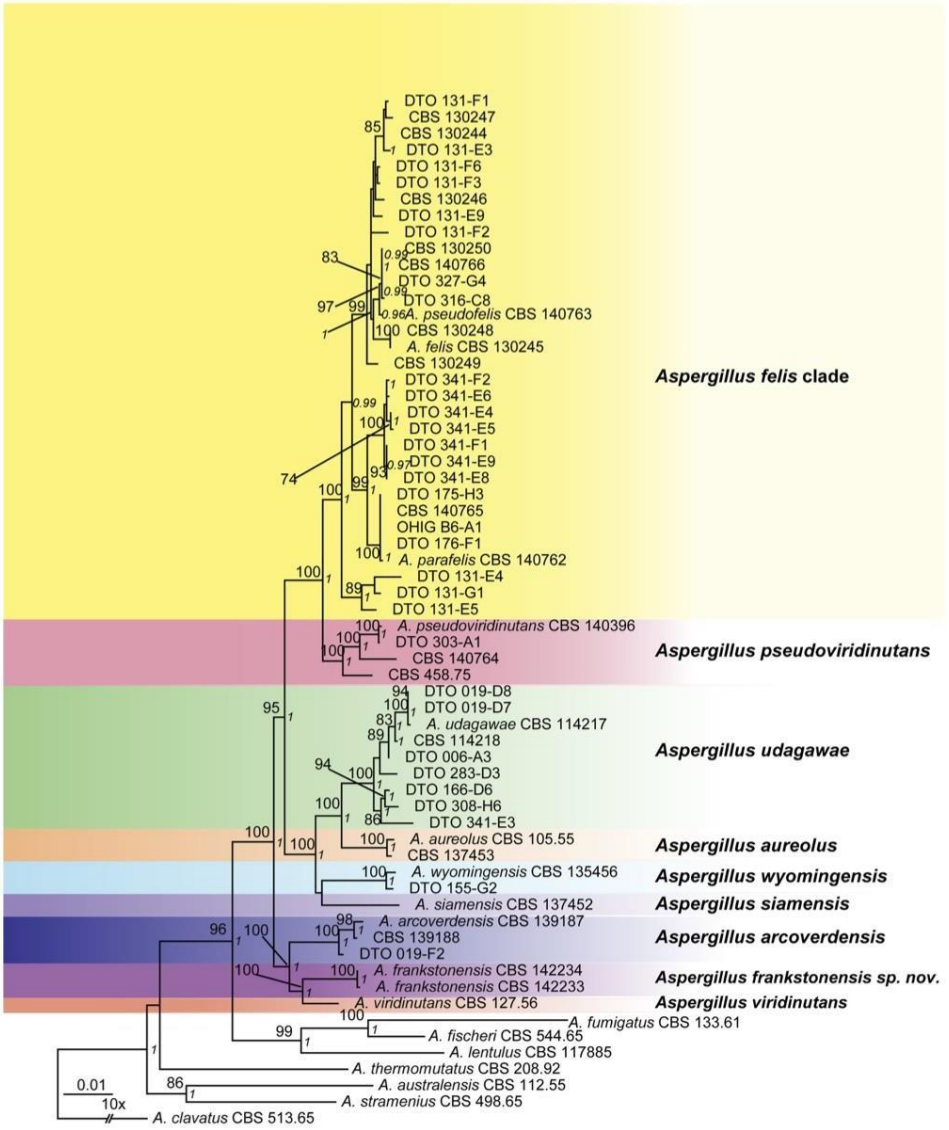


Fig 1. *Aspergillus viridinutans* species complex combined phylogenetic tree. Tree based on sequencing of *Mcm7*, *BenA*, *Act*, *RPB2*, *CaM* genes (phylogeny model Kimura 2, gamma distribution, 1000 bootstrap replicates with Bayesian method posterior probability values in italics). Isolates previously described as *A. felis*, *A. parafelis* and *A. pseudofelis* are listed here under the one grouping "*A. felis*-clade".

https://doi.org/10.1371/journal.pone.0181660.g001

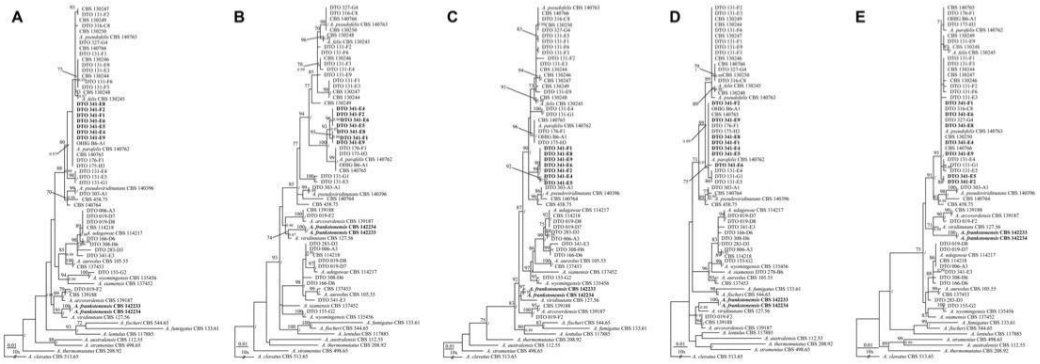


Fig 2. *Aspergillus viridinutans* species complex individual gene phylogenetic trees. All phylogenies made with gamma distribution and 1000 bootstrap replicates with Bayesian method posterior probability values in italics. A = *Act* (phylogeny model General Time Reversible); B = *BenA* (phylogeny model Kimura 2); C = *CaM* (phylogeny model Tamura-Nei); D = *Mcm7* (phylogeny model Kimura 2); E = *RPB2* (phylogeny model General Time Reversible).

https://doi.org/10.1371/journal.pone.0181660.g002

341-E4, DTO 341-E5, DTO 341-F1, DTO 341-E9, DTO 341-E8) were also shown to be accommodated in the AVSC and ASF. The results of the analysis of the combined dataset (Fig 1) showed that these isolates were with high statistical support (99% bs, 1.00 pp) most closely related to the type of *A. parafelis* CBS 140762. The individual and combined phylogenies also showed that isolate CBS 458.75 is accommodated in the AVSC, most closely related to *A. pseudoviridinutans* isolates in five of the six trees generated (*Act* 70% bs, 0.96 pp; *CaM* 96% bs, 1.00 pp; *Mcm7* 89% bs 1.00 pp; *RPB2* 91% bs, 0.98 pp; combined 100% bs, 1.00 pp). Isolates DTO 131-E4, DTO 131-E5 and DTO 131-G1 formed a separate clade to other *A. felis* isolates in the *Act* (98% bs, 1.00 pp), *Mcm7* (86% bs, <0.95 pp), *RPB2* (<70% bs, 0.96 pp) and combined trees (89%, 1.00 pp). The *BenA* tree showed DTO 131-G1 and DTO 131-E5 were in the same clade (100% bs, 1.00 pp) and basal to the *A. felis*-clade, while DTO 131-E4 was more closely related to the type of *A. felis* (CBS 130245) (70% bs, 0.99 pp). In the *CaM* tree, DTO 131-E4 and DTO 131-G1 were in the same clade (100% bs, 1.00 pp) but their position in the *A. felis*-clade was unresolved, and DTO 131-E5 was positioned in a clade with moderate statistical support (73% bs, < 0.95 pp) related to the types for *A. pseudofelis* (CBS 140763) and *A. felis* (CBS 130245).

Taxonomy

Morphological and physiological characterization

Species description of *A. frankstonensis* (CBS 142233 = DTO 341-E7 = IBT 34172; CBS 142234 = DTO 341-F3 = IBT 34204)

Aspergillus frankstonensis Talbot et al 2017, **sp. nov.** MycoBank 819986. Fig 3.

Etymology. Named after Frankston, Australia, the collection location of the type strain. This town in Victoria, Australia was also the location where *Aspergillus viridinutans s. str.* was first isolated [16].

Diagnostic characteristics. *Aspergillus frankstonensis* belongs in *Aspergillus* subgenus *Fumigati* section *Fumigati* and is phenotypically similar to other members of the AVSC as it is generally slow to sporulate and thermophilic. The species is phylogenetically most closely

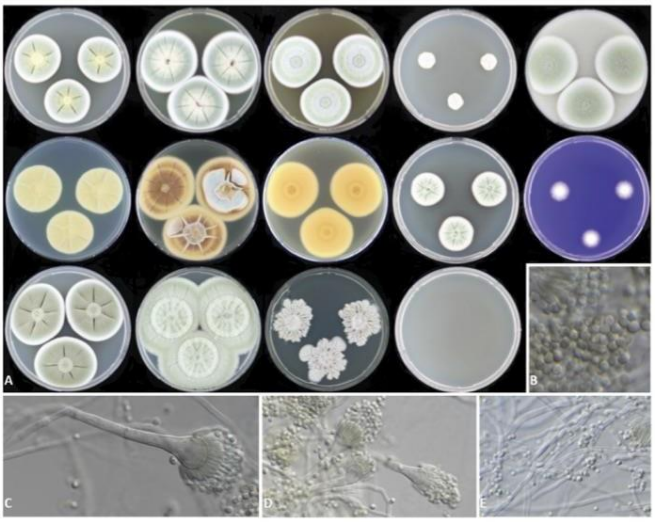


Fig 3. *Aspergillus frankstonensis* CBS 142233; CBS 142234. (A) Colonies grown at 25˚C for 7 days, from left to right (top row) CYA, YES, MEA, CYAS, OA; middle row CYA reverse, YES reverse, MEA reverse, DG18, CREA; bottom CYA grown at 30˚C, 37˚C, 45˚C, 50˚C. (B) Conidia (C—D) Conidiophores (E) Hyphae.

https://doi.org/10.1371/journal.pone.0181660.g003

related to *A. virdinutans s. str.* but differs from this species by its ability to sporulate well at 37˚C and grow at 45˚C.

Specimen examined. **Australia** (latitude 38.1414˚S, longitude 145.1225˚E), from soil, collection date May 7th 2015, J. Talbot & V. Barrs, (holotype **CBS-H-22969**, culture ex-type CBS 142233 = DTO 341-E7; **Australia**, identical collection information as CBS 142233, CBS 142234 = DTO 341-F3).

Description (Fig 3)

Colony diam, 7 days, 25˚C (mm): CYA 30–43; MEA 28–34; YES 42–55; DG18 40–45; CYAS 18–22; OAT 40–43; DG18 20–24; CREA 10–27, poor growth, no acid production. Other incubation temperatures: CYA 30˚C 42–53; CYA 37˚C 45–56; CYA 45˚C 25; CYA 50˚C no growth; optimum growth temperature 37˚C, maximum between 45 and 50˚C.

Macromorphology: CYA 25˚C, 7 days: colony sulcate, radiating and concentric patterns; sporulation moderate; colony texture velvety to floccose; conidia yellow-green in centre of colony, dull green towards edge; non-sporulating edge 2 mm; mycelium white to yellow; soluble pigment present, yellow; exudate present as yellow droplets; margin regular; reverse yellow.

MEA 25˚C, 7 days: colony slightly raised, sulcate, radiating; sporulation poor; colony texture velvety to floccose; mycelium white to pale yellow; conidia pale to bright green; exudate present as clear to yellow droplets; reverse yellow to orange. YES 25˚C, 7 days: colony sulcate; sporulation moderate; mycelium white; conidia *en masse* pale-dull green; non-sporulating edge 3 mm; soluble pigments orange; exudate absent; reverse brown and yellow. DG18 25˚C, 7 days: colony sulcate; sporulation moderate, conidia dull green, mycelium white with pink tinge; soluble pigment present, pink to yellow; exudate absent, reverse yellow to brown. OA

25˚C, 7 days: colony elevated; sporulation moderate; colony texture floccose; mycelium white; conidia pale green; soluble pigment absent; exudate absent; sclerotia absent; reverse yellow.

Micromorphology: Conidial heads columnar, uniseriate. Stipes hyaline, smooth walled, 60–130 × 4–5 μm. Vesicles subglobose, up to 12.5 μm in diameter. Phialides ampulliform, 6–8 × 2–3 μm, covering ~75% of the head. Conidia globose, smooth, hyaline 2 × 2–3 μm; average width/length = 0.95, n = 40; Hülle cells absent.

Occurrence. This species has been found in the soil at a recreational reserve, Upper Sweetwater Creek

Reserve in Frankston, Victoria, Australia, 38.1580˚ S, 145.1350˚ E.

Genbank accession numbers: CBS 142233: KY808756 (ITS); KY808594 (*BenA*); KY808724 (*CaM*); KY808948 (*RPB2*); KY808549 (*Act*); KY808901 (*Mcm7*). CBS 142234: KY808761(ITS); KY808599 (*BenA*); KY808729 (*CaM*); KY808953 (*RPB2*); KY808554 (*Act*); KY808906 (*Mcm7*).

Secondary metabolite production

Both isolates produced viriditoxin, apolar indole-alkaloids and three new compounds given the temporary names of DOLD, RAIMO and CALBO, based on their unique UV spectra. The CALBO compounds had an absorption maximum at 343 nm quite similar to calbistrins. One of the isolates (CBS 142234) was also observed to produce two chrysogine precursors, a unique apolar indol alkaloid and an additional three new compounds given the temporary names of OKAM, USOC and COT based on their UV spectra. The other isolate (CBS 142233) produced aszonapyrone A & B, a chrysogine precursor, apolar indolalkaloids and an additional two compounds given the temporary name of HITO and BRUDA. *Aspergillus frankstonensis* shares viriditoxin production with *A. viridinutans*, and aszonapyrones with several *Aspergillus* section *Fumigati* species.

Antifungal susceptibilities

Antifungal susceptibility results are summarized in Table 2 for two *A. frankstonensis* isolates and seven *A. felis*-clade isolates (6 environmental, 1 clinical). There was no observed activity of ITZ against one environmental *A. felis*-clade isolate.

Table 2. Antifungal susceptibility results for two *A. frankstonensis* isolates, six *A. felis*-clade isolates from environmental soil and one *A. felis*-clade clinical isolate (cat). MIC/MEC (μg/mL) values reflect the number of isolates within the specific cut-off value.

Drug	Species	MIC/MEC (μg/mL) distribution among tested isolates												
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	>16	GM
AMB*	*A. frankstonensis*				1			1						0.35
	A. felis-clade							1	5	1				2.00
ITZ*	*A. frankstonensis*				1		1							0.24
	A. felis-clade							4	2				1	1.26
VCZ*	*A. frankstonensis*							1		1				2.00
	A. felis-clade							1	2	4				2.69
POS*	*A. frankstonensis*					1	1							0.35
	A. felis-clade						1	6						0.90
CSP†	*A. frankstonensis*	1	1											0.02
	A. felis-clade	5	1	1										0.02

*MIC, minimum inhibitory concentration;

†MEC, minimum effective concentration

https://doi.org/10.1371/journal.pone.0181660.t002

Table 3. Intra- and interspecific mating results among environmental *A. felis*-clade isolates.

	MAT1-1 strain						
	***A. felis*-clade**	***A. felis*-clade**	***A. felis*-clade**	***A. felis*-clade**	***A. felis*-clade**	***A. felis***	***A. felis***
MAT1-2 strain	**DTO 341-F1**	**DTO 341-E6**	**DTO 341-E4**	**DTO 341-E8**	**DTO 341-E9**	**DTO 131-E3**	**DTO 131-E5**
A. felis-clade **DTO 341-F2**	+	+*	-	-	+	+	+
***A. felis*-clade** **DTO 341-E5**	+*	+*	-	-	-	-	-
A. felis **DTO 131-E4**	-	+*	+	-	-	-	-
A. wyomingensis **CCF 4416**	-	+	-	-	-	-	-
A. frankstonensis **DTO 341-F3**	-	-	-	-	-	-	-

- no cleistothecia or ascospores;

+ cleistothecia production;

*fertile ascospores

https://doi.org/10.1371/journal.pone.0181660.t003

Mating type analysis

Both isolates of *A. frankstonensis* sp. nov. were MAT1-2. All pairings with MAT1-2 isolates of other AVSC species were negative (supplementary Table 2). Both mating types MAT1-1 (n = 5) and MAT1-2 (n = 2) were found amongst *A. felis*-clade environmental isolates. Positive intra-species and inter-species matings between opposite mating types (Table 3) produced clusters of white to creamish cleistothecia along the barrage zone that contained lenticular ascospores with two prominent equatorial crests and an echinulate convex surface. Ascospores from three *A. felis*-clade intra-species and one inter- species pairing with *A. felis* were fertile and from one pairing with *A. wyomingensis* were infertile (Fig 4). No growth was seen from parental strains.

Taxonomic notes

Aspergillus frankstonensis has some unique morphological characteristics that can be used to further distinguish it from its closest AVSC relatives (including *A. viridinutans s. str.*, *A. arcoverdensis* and *A. udagawae*). Grossly, when grown on CYA at 25˚C in the dark for 7 days, *A. frankstonensis* has a smaller colony diameter than *A. arcoverdensis* and *A. udagawae* (*A. frankstonensis* 30–43 mm; *A. arcoverdensis* 56–58 mm; *A. udagawae* 82–85 mm), and a

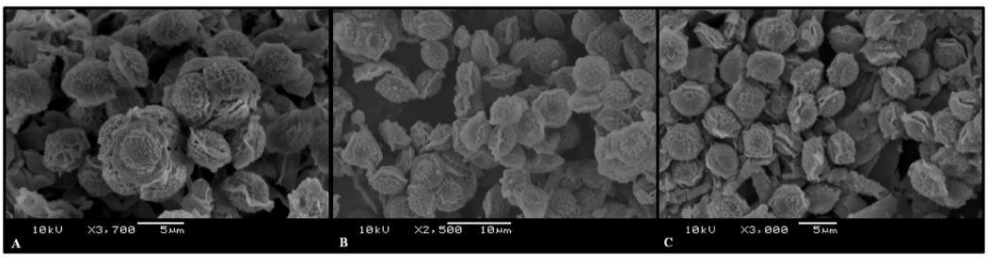


Fig 4. Ascospores from mating experiments with *A. felis*-clade isolates. A = *A. felis*-clade (DTO 341-E6) X *A. felis*-clade (DTO 341-E5); B = *A. felis*-clade (DTO 341-E6) X *A. wyomingensis* (CCF 4416; C = *A. felis*-clade (DTO 341-F2) X *A. felis* (DTO 131-E3).

https://doi.org/10.1371/journal.pone.0181660.g004

larger colony diameter than *A. viridinutans* (28–40 mm) [14, 33]. On MEA *A. frankstonensis* has pale to bright green conidia on white to pale yellow mycelium, whereas its closely related species range from yellowish white (*A. arcoverdensis*) to gray green (*A. viridinutans*) to dull green (*A. udagawae*) [33]. Microscopically, the conidial head of *A. frankstonensis* (60–130 × 4–5 μm) differs in size compared to *A. arcoverdensis* (82–110 × 22.5–30 μm), *A. viridinutans* (50 × 30 μm) and *A. udagawae* (95–145 × 20–50 μm) [33]. It differs from *A. fumigatus* by its inability to grow at 50˚C.

Discussion

Our findings suggest that human and animal exposure to pathogenic *Aspergillus* species in Australia is not uncommon since 25% of all ASF isolates were pathogenic species. As expected, the predominant *Aspergillus* species isolated was *A. fumigatus s. str.* and this species remains the most common cause of aspergillosis in humans and animals. However, the incidence of aspergillosis due to other species in ASF is increasing, reported between 3 to 5% of aspergillosis cases in human patients [34–38].

In soil from residential environments we found the human and animal pathogens *A. fumigatus*, *A. fischeri*, *A. laciniosus*, *A. lentulus*, *A. fumisynnematus*, *A. hiratsukae* and *A. thermomutatus* (syn. *N. pseudofischeri*). Four of these (*A. fumigatus.*, *A. fischeri*, *A. lentulus* and *A. thermomutatus*) cause IFI in cats [4, 10]. Isolation of these known feline pathogens from soil in areas cats had access to supports that the source of feline infections is environmental. *A. felis* conidia have also been found in air [4], making infection of cats possible via disruption of soil or wind dispersal, such as could occur through the natural feline behaviours of digging, sniffing and grooming. Given that infection often occurs in brachycephalic purebred cats of Persian lineage [4, 9], an immunogenetic defect may predispose cats to disease [10, 39].

Interestingly, we only isolated AVSC species from soil from Frankston, but not from residential environments, cat litter or cat food. *A. viridinutans s. str.*, was discovered in Frankston in 1954 in rabbit dung and sandy soil [16]. This suggests a possible ecological niche for AVSC species at this site associated with faunae, flora and associated soils [40], or local processes such as bush regeneration and back-burning which may remove competing microbiota [41, 42]. AVSC species have been found to be abundant in other specific regions, including a coal mining reclamation site in Wyoming, USA [14].

The most abundant AVSC species isolated from Frankston were in the *A-felis*-clade, closely related to the type of *A. parafelis*, CBS 140762, which is a clinical isolate from the oropharyngeal exudate of a human [8]. Here, we also identified another closely related isolate, OHIGB6-A1, which was cultured from a cat with sino-orbital aspergillosis.

Our soil isolation technique was adapted from two previous studies [14, 18]. As *A. felis* is a heterothallic species producing heat resistant ascospores, we aimed to recover activated ascospores by heat treating soil. Soil processed without heat did not recover any members of the AVSC. Thus, the ascospores of these species are present in soil and heat activation appears to be an important surveillance technique for heterothallic AVSC species.

Following their polyphasic taxonomical analysis of 11 AVSC isolates, Sugui et al (2014) described three phylogenetically closely related but separate species within the *A. felis*-clade; the already described *A. felis* [4] and two new species, *A. parafelis* and *A. pseudofelis*. We investigated the phylogenetic relationships of 56 clinical and environmental AVSC isolates (Fig 1). Our phylogenetic data, based on the single gene and a combined dataset of six genes demonstrated that *A. felis* and *A. pseudofelis* are the same species. The distinctiveness of *A. parafelis* is questionable due to the positioning of a clade of previously described *A. felis* isolates DTO 131 E4, DTO 131-E5 and DTO 131-G1 [4]. These isolates have in the combined dataset a basal

position to the *A. felis*-clade, but the position of these isolates in the single gene phylogenies isn't congruent. This data indicates that *A. parafelis* is also conspecific with *A. felis*, when the gene concordance phylogenetic species recognition concept is applied. This is supported by mating experiments and isolates from different lineages are all able to mate [8]. We demonstrated fertile mating between *A. felis* (DTO 131-F4) and an *A. felis*-clade isolate (DTO 341-E6), which is closely related to the type strain for *A. parafelis* (CBS 140762).

Our phylogenetic data also showed that isolate CBS 458.75 is phylogenetically most closely related to *A. pseudoviridinutans*. Investigation into exometabolite production will further determine the relationship, however preliminary studies have shown CBS 458.75 is able to produce some of the same exometabolites that *A. pseudoviridinutans* strains (DTO 303-A1, NRRL 6106) produce, including antafumicins, clavatols, fumigatin, VERN and pseurotins.

Here we reported the discovery of a new AVSC species, *A. frankstonensis*, which is of unknown pathogenicity. However, the ability to sporulate at 37˚C indicate pathogenic potential [1]. Although MICs of most antifungals tested were generally low, one *A. frankstonensis* isolate had a high MIC of VCZ (4 ug/mL).

We performed AVSC inter-species mating experiments on *A. frankstonensis* sp. nov. as other AVSC members have been reported to mate with other species in the complex [8]. *A. frankstonensis* inter-species mating were negative, further confirming its status as a distinct species. Intraspecies mating tests could not be performed as we had only one mating type for *A. frankstonensis*. Heterothallism (sexual reproduction) allows genetic recombination and has the potential to increase fitness. This may be beneficial for adaptation to environmental conditions, and may also contribute to drug resistance [43]. Interestingly, the majority of the 19 known pathogenic fungi from the genus *Aspergillus* are also heterothallic, with the exception of some doubtful species, *A. beijingensis*, *A. qizutongi* and *A. wanduanglii* [44]. However, many heterothallic species of unknown pathogenicity also exist.

Recent studies have demonstrated that the small molecule extrolite (secondary metabolite), profiles of ASF species can determine the relatedness and identification of a species [45], and may also predict the potential pathogenicity of a new species where only environmental isolates have been discovered [1]. Extrolite production by AVSC environmental isolates in this study shared similarities with other members in the complex. All isolates were shown to produce viriditoxin, which is produced by all other members of the AVSC and one other ASF species [1], therefore its link to pathogenicity is unknown. The only other ASF species reported to produce viriditoxin is *A. denticulatus* [46]. There were some secondary metabolite differences between the two *A. frankstonensis sp. nov.* isolates from Frankston soil. One isolate produced aszonapyrones and chrysogine precursors which may be associated with pathogenicity [47, 48]. Aszonapyrones have antibacterial properties [47]; and chrysogine is an alkaloid [48]. There was a notable difference in the degree of sporulation between the two isolates with one demonstrating poor sporulation. This may account for the differences in extrolite profiles. However, phylogenetically they are the same species with no nucleotide differences between them, based on a number of targeted genes. Novel extrolites were also produced by these isolates, some of which were shared. These unique extrolites may be produced for fungal competitiveness in the primary habitat of this fungal species. Further analysis of the novel compounds and secondary metabolite profiling of other members of the AVSC will be undertaken by the authors for further comparison between environmental and pathogenic strains.

Conclusions

The risk of exposure to pathogenic species in ASF in Australia appears to be high. The risk of direct environmental exposure to the AVSC in areas where humans and cats co-habitate in

Australia, however appears to be low. There was no evidence of an environmental reservoir of these organisms in the homes of any cats diagnosed with aspergillosis. Detection of AVSC organisms from only one location suggests a niche for these species that favours specific environmental conditions. *A. frankstonensis* sp. nov is an interesting new species in ASF that is closely related to known human and animal pathogens and possesses some virulent characteristics including growth at 37˚C and a high MIC of voriconazole. It also produces unique secondary metabolites that require further investigation.

Supporting information

S1 Fig. Phylogenetic trees of 104 environmental isolates and type strains for *A.* section *Fumigati* based on *BenA* sequencing (phylogeny model Kimura 2, gamma distribution, 1000 bootstrap replicates).
(ZIP)

S1 Table. *Aspergillus* sect. *Fumigati* species isolated from air and diluted, heat-treated soil from eight Australian properties where cats were domiciled with confirmed feline upper respiratory tract aspergillosis due to AVSC and one National Park site. (#), number of isolates found at property.
(PDF)

S2 Table. AVSC species isolates that were paired with *A. frankstonensis* sp. nov. DTO 341-F3 (MAT1-2).
(PDF)

Acknowledgments

The authors wish to thank Martin Meijer and Tineke van Doorn at Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands for technical assistance.

Author Contributions

Conceptualization: Julia A. Beatty, Vanessa R. Barrs.

Data curation: Jessica J. Talbot, Vanessa R. Barrs.

Formal analysis: Jessica J. Talbot, Jos Houbraken, Jens C. Frisvad, Sue Lindsay.

Funding acquisition: Jessica J. Talbot, Vanessa R. Barrs.

Investigation: Jessica J. Talbot, Jos Houbraken, Sarah E. Kidd, Sue Lindsay, Vanessa R. Barrs.

Methodology: Jessica J. Talbot, Jos Houbraken, Jens C. Frisvad, Sarah E. Kidd, John Pitt, Vanessa R. Barrs.

Project administration: Jessica J. Talbot.

Resources: Jessica J. Talbot, Jos Houbraken, Jens C. Frisvad, Robert A. Samson, Julia A. Beatty, Vanessa R. Barrs.

Supervision: Jos Houbraken, Jens C. Frisvad, Robert A. Samson, Sarah E. Kidd, John Pitt, Vanessa R. Barrs.

Validation: Jessica J. Talbot, Jos Houbraken, Robert A. Samson, Vanessa R. Barrs.

Visualization: Jessica J. Talbot, Vanessa R. Barrs.

Writing – original draft: Jessica J. Talbot, Jos Houbraken, Vanessa R. Barrs.

Writing – review & editing: Jessica J. Talbot, Jos Houbraken, Jens C. Frisvad, Robert A. Samson, Sarah E. Kidd, John Pitt, Sue Lindsay, Julia A. Beatty, Vanessa R. Barrs.

References

1. Frisvad JC, Larsen TO. Extrolites of *Aspergillus fumigatus* and other pathogenic species in *Aspergillus* section *Fumigati*. Front Microbiol. 2016; 6: Art. 1485. https://doi.org/10.3389/fmicb.2015.01485 PMID: 26779142
2. Barrs VR, Beatty JA, Dhand NK, Talbot JJ, Bell E, Abraham LA, et al. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. Vet J. 2014; 201(2): 215–222. https://doi.org/10.1016/j.tvjl.2014.02.020 PMID: 24685469
3. Vinh DC, Shea YR, Sugui JA, Parrilla-Castellar ER, Freeman AF, Campbell JW, et al. Invasive aspergillosis due to *Neosartorya udagawae*. Clin Infect Dis. 2009; 49(1): 102–111. https://doi.org/10.1086/599345 PMID: 19489714
4. Barrs VR, van Doorn TM, Houbraken J, Kidd SE, Martin P, Pinheiro MD, et al. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. PLoS One. 2013; 8(6): e64871. https://doi.org/10.1371/journal.pone.0064871 PMID: 23798996
5. Alvarez-Pérez S, Mellado E, Serrano D, Blanco JL, Garcia ME, Kwon M, et al. Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. J Med Microbiol. 2014; 63(Pt 4): 617–619. https://doi.org/10.1099/jmm.0.068502-0 PMID: 24469682
6. Peláez T, Alvarez-Pérez S, Mellado E, Serrano D, Valerio M, Blanco JL, et al. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. J Med Microbiol. 2013; 62(Pt 3): 474–478. https://doi.org/10.1099/jmm.0.044867-0 PMID: 23161769
7. Coelho D, Silva S, Vale-Silva L, Gomes H, Pinto E, Sarmento A, et al. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. Med Mycol. 2011; 49(7): 755–759. https://doi.org/10.3109/13693786.2011.556672 PMID: 21299372
8. Sugui JA, Peterson SW, Figat A, Hansen B, Samson RA, Mellado E, et al. Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. J Clin Microbiol. 2014; 52 (10): 3707–3721. https://doi.org/10.1128/JCM.01704-14 PMID: 25100816
9. Barrs VR, Halliday C, Martin P, Wilson B, Krockenberger M, Gunew M, et al. Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. Vet J. 2012; 191(1): 58–64. https://doi.org/10.1016/j.tvjl.2011.02.009 PMID: 21388842
10. Barrs VR, T JJ. Feline aspergillosis. Vet Clin North Am Small Anim Pract. 2014; 44(1): 51–73. https://doi.org/10.1016/j.cvsm.2013.08.001 PMID: 24268333
11. Whitney BL, Broussard J, Stefanacci JD. Four cats with fungal rhinitis. J Feline Med Surg. 2005; 7(1): 53–58. https://doi.org/10.1016/j.jfms.2004.02.004 PMID: 15686974
12. Furrow E, Groman RP. Intranasal infusion of clotrimazole for the treatment of nasal aspergillosis in two cats. J Am Vet Med Assoc. 2009; 235(10): 1188–1193. https://doi.org/10.2460/javma.235.10.1188 PMID: 19912040
13. Kano R, Takahashi T, Hayakawa T, Yamaya Y, Hasegawa A, Kamata H. The first case of feline sinonasal aspergillosis due to *Aspergillus fischeri* in Japan. J Vet Med Sci. 2015; 77(9): 1183–1185. https://doi.org/10.1292/jvms.14-0454 PMID: 25947561
14. Nováková A, Hubka V, Dudová Z, Matsuzawa T, Kubátová A, Yaguchi T, et al. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. Fungal Diversity. 2014; 64(1): 253–274.
15. Gonçalves VN, Cantrell CL, Wedge DE, Ferreira MC, Soares MA, Jacob MR, et al. Fungi associated with rocks of the Atacama Desert: taxonomy, distribution, diversity, ecology and bioprospection for bioactive compounds. Environ Microbiol. 2016; 18(1): 232–245. https://doi.org/10.1111/1462-2920.13005 PMID: 26235221
16. McLennan EI, Ducker SC, Thrower LB. New soil fungi from Australian heathland: *Aspergillus*, *Penicillium*, *Spegazzinia*. Aust J Bot. 1954; 2: 355–364.
17. Kidd SE, Ling LM, Meyer W, Orla Morrissey C, Chen SC, Slavin MA. Molecular epidemiology of invasive aspergillosis: lessons learned from an outbreak investigation in an Australian hematology unit. Infect Control Hosp Epidemiol. 2009; 30(12): 1223–1226. https://doi.org/10.1086/648452 PMID: 19848605
18. Houbraken J, Samson RA. Standardization of methods for detecting heat-resistant fungi. Adv Exp Med Biol. 2006; 571: 107–111. https://doi.org/10.1007/0-387-28391-9_5 PMID: 16408595

19. Samson RA, Houbraken J, Thrane U, Frisvad JC. Food and Indoor fungi. Utrecht: Centraalbureau voor Schimmelcultures; 2010.
20. White TJ, Bruns T, Lee SJ T W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A. G DH, Sninsky J. J. & White T. J., editor. PCR Protocols A Guide to Methods and Applications. New York: Academic Press; 1990. pp. 315–322.
21. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol. 1995; 61(4): 1323–1330. PMID: 7747954
22. National Centre for Biotechnology Information. Basic Local Alignment Search Tool (BLAST): Standard Nucleotide BLAST 2016 [cited 2016 15 July]. http://blast.ncbi.nlm.nih.gov/Blast.cgi.
23. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013; 30: 2725–2729. https://doi.org/10.1093/molbev/mst197 PMID: 24132122
24. Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia. 2005; 97(6): 1316–1329. PMID: 16722222
25. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics Applications Note. 2006; 22(21): 2688–2690.
26. Ronquist F, Huelsenbecj J, Teslenko M. MrBayes version 3.2.1 http://mrbayes.sourceforge.net/2011 [cited 2017 January].
27. Frisvad JC, Samson RA. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud Mycol 2004; 49: 1–173.
28. Hocking AD, Pitt JI. Fungi and Food Spoilage. Third ed. New York: Springer Science + Business Media LLC; 2009.
29. Smedsgaard J. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A. 1997; 760(2): 264–270. PMID: 9062989
30. Nielsen ML, Nielsen JB, Rank C, Klejnstrup ML, Holm DK, Brogaard KH, et al. A genome-wide polyketide synthase deletion library uncovers novel genetic links to polyketides and meroterpenoids in Aspergillus nidulans. FEMS Microbiol Lett. 2011; 321(2):157–66. https://doi.org/10.1111/j.1574-6968.2011.02327.x PMID: 21658102
31. Talbot JJ, Kidd SE, Martin P, Beatty JA, Barrs VR. Azole resistance in canine and feline isolates of Aspergillus fumigatus. Comp Immunol Microbiol Infect Dis. 2015; 42: 37–41. https://doi.org/10.1016/j.cimid.2015.08.002 PMID: 26387063
32. O'Gorman CM, Fuller HT, Dyer PS. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. Nature. 2009; 457: 471–475. https://doi.org/10.1038/nature07528 PMID: 19043401
33. Matsuzawa T, Takaki GMC, Yaguchi T, Okada K, Abliz P, Gonoi T, et al. *Aspergillus arcoverdensis*, a new species of *Aspergillus* section *Fumigati* isolated from caatinga soil in State of Pernambuco, Brazil. Mycoscience. 2015; 56(2): 123–131.
34. Alastruey-Izquierdo A, Mellado E, Peláez T, Pemán J, Zapico S, Alvarez M, et al. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). Antimicrob Agents Chemother. 2013; 57(7): 3380–3387. https://doi.org/10.1128/AAC.00383-13 PMID: 23669377
35. Balajee SA, Kano R, Baddley JW, Moser SA, Marr KA, Alexander BD, et al. Molecular identification of *Aspergillus* species collected for the transplant-associated infection surveillance network. J Clin Microbiol. 2009; 47(10): 3138–3141. https://doi.org/10.1128/JCM.01070-09 PMID: 19675215
36. Negri CE, Gonçalves SS, Xafranski H, Bergamasco MD, Aquino VR, Castro PT, et al. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. J Clin Microbiol. 2014; 52(10): 3633–3640. https://doi.org/10.1128/JCM.01582-14 PMID: 25078909
37. Sabino R, Veríssimo C, Parada H, Brandão J, Viegas C, Carolino E, et al. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. Med Mycol. 2014; 52(5):519–529. https://doi.org/10.1093/mmy/myu006 PMID: 24847040
38. Escribano P, Peláez T, Muñoz P, Bouza E, Guinea J. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? Antimicrob Agents Chemother. 2013; 57(6):2815–2820. https://doi.org/10.1128/AAC.02487-12 PMID: 23629706
39. Whitney JL, Krockenberger MB, Day MJ, Beatty JA, Dhand NK, Barrs VR. Immunohistochemical Analysis of Leucocyte Subsets in the Sinonasal Mucosa of Cats with Upper Respiratory Tract Aspergillosis. J Comp Pathol. 2016; 155(2–3): 130–140. https://doi.org/10.1016/j.jcpa.2016.08.001 PMID: 27576043
40. Sylvain ZA, Wall DH. Linking soil biodiversity and vegetation: implications for a changing planet. Am J Bot. 2011; 98(3): 517–527. https://doi.org/10.3732/ajb.1000305 PMID: 21613143

41. Greene RSB, Chartres CJ, Hodgkinson KH. The effect of fire on the soil in a degraded semi-arid woodland. I. Cryptogram cover and physical and micromorphological properties. Aust J Soil Res. 1990; 28: 755–777.

42. Eldridge DJ, Greene RSB. Microbiotic soil crusts: a review of their roles in soil and ecological processes in the rangelands of Australia. Aust J Soil Res. 1994; 32: 389–415.

43. Ni M, Feretzaki M, Sun S, Wang X, Heitman J. Sex in Fungi. Annu Rev Genet. 2011; 45: 405–430. https://doi.org/10.1146/annurev-genet-110410-132536 PMID: 21942368

44. Samson RA, Hong S, Peterson SW, Frisvad JC, Varga J. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. Stud Mycol. 2007; 59: 147–203. https://doi.org/10.3114/sim.2007.59.14 PMID: 18490953

45. Panda A, Ghosh AK, Mirdha BR, Xess I, Paul S, Samantaray JC, et al. MALDI-TOF mass spectrometry for rapid identification of clinical fungal isolates based on ribosomal protein biomarkers. J Microbiol Methods. 2015; 109: 93–105. https://doi.org/10.1016/j.mimet.2014.12.014 PMID: 25541362

46. Hong SB, Shin HD, Hong J, Frisvad JC, Nielsen PV, Varga J, et al. New taxa of *Neosartorya* and *Aspergillus* in *Aspergillus* section *Fumigati*. Antonie Van Leeuwenhoek. 2008; 93(1–2): 87–98. https://doi.org/10.1007/s10482-007-9183-1 PMID: 17610141

47. Gomes NM, Bessa LJ, Buttachon S, Costa PM, Buaruang J, Dethoup T, et al. Antibacterial and antibiofilm activities of tryptoquivalines and meroditerpenes isolated from the marine-derived fungi *Neosartorya paulistensis*, *N. laciniosa*, *N. tsunodae*, and the soil fungi *N. fischeri* and *N. siamensis*. Mar Drugs. 2014; 12(2): 822–839. https://doi.org/10.3390/md12020822 PMID: 24477284

48. Pildain MB, Frisvad JC, Vaamonde G, Cabral D, Varga J, Samson RA. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. Int J Syst Evol Microbiol. 2008; 58(Pt 3): 725–735. https://doi.org/10.1099/ijs.0.65123-0 PMID: 18319485

Appendix 3: Identification of pathogenic *Aspergillus* isolates from captive birds in Australia

This is a pre-copy edited, author-produced version of an article accepted for publication in *Medical Mycology* following peer review. The version of record Talbot JJ, Thompson P, Vogelnest L, Barrs VR. Identification of pathogenic *Aspergillus* isolates from captive birds in Australia. *Medical Mycology*, 2017; myx137, is available online at https://doi.org/10.1093/mmy/myx137.

ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2017, 0, 1–4
doi: 10.1093/mmy/myx137
Advance Access Publication Date: 0 2017
Brief Report

Brief Report

Identification of pathogenic *Aspergillus* isolates from captive birds in Australia

Jessica J. Talbot[1], Paul Thompson[2], Larry Vogelnest[2] and Vanessa R. Barrs[1,*]

[1]Sydney School of Veterinary Science, Faculty of Science, and Marie Bashir Institute of Infectious Diseases & Biosecurity, The University of Sydney, NSW 2006 Australia and [2]Taronga Conservation Society Australia, Mosman, NSW 2088 Australia

*To whom correspondence should be addressed. Vanessa R. Barrs, BVSc(hons) PhD MVetClinStud FANZCVS, University Veterinary Teaching Hospital, 65 Parramatta Rd, Camperdown NSW 2050 Australia. Tel: +612 9351 3436; Fax: +612 9351 7436; E-mail: vanessa.barrs@sydney.edu.au

Received 14 August 2017; Revised 19 October 2017; Accepted 2 November 2017; Editorial Decision 23 October 2017

Abstract

Aspergillosis is a major cause of severe respiratory disease in birds. The prevalence of cryptic section *Fumigati* and other non–*Aspergillus fumigatus* species as causative agents is unknown. Species identity was determined in 30 isolates from affected birds from zoos, pet birds and poultry by PCR of the ITS1-5.8S-ITS2 and partial β-tubulin genes. The most prevalent isolate was *A. fumigatus* sens. str. in 87% (26) cases. Other *Aspergillus* species were identified in 13% (4) cases, including *A. restrictus* (1), *A. flavus* sens. str. (2), and *A. nidulans*-clade (1). This is the first report of *A. restrictus* causing avian disease.

Key words: avian aspergillosis, avian mycoses, Australia.

Introduction

Avian aspergillosis, first reported in the 1800s,[1] is a significant cause of mortality among captive birds worldwide. It most commonly causes disease within the respiratory tract, and can progress to disseminated invasive aspergillosis. Two forms of lesions have been described; a granulomatous deep nodular form affecting non-aerated parenchyma, and a pyogranulomatous nonencapsulated superficial diffuse form involving lungs and serosae.[2]

Captive birds are highly susceptible to aspergillosis due to environmental factors (increased burden of fungal spores), host immune factors (immunosuppression from disease, stress, or therapeutics), and unique respiratory anatomy.[1,2] Anatomical differences to mammals that enable unique airflow in birds include nonexpansile and unlobed lungs with no pleural cavity, looped parabronchi with bi-directional air flow capillaries instead of terminal ending alveoli, and cranial and caudal air sacs that extend the bronchial system beyond the lung to pneumatize bones and enable fresh airflow during inspiration and expiration.[3] Infection occurs when innate defenses cannot eliminate spores in parabronchial air capillaries, resulting in fungal plaques that can penetrate surrounding tissues.[2] Despite a range of antifungal therapeutic treatment options, infections are often fatal.[2]

The clinical importance of fungal molecular identification techniques has been demonstrated for human and nonavian animal species with aspergillosis, with cryptic section *Fumigati* species increasingly identified, prompting epidemiological studies.[4–9] We previously identified isolates causing respiratory aspergillosis in cats and dogs and found that disease in dogs, like humans is predominantly caused by *A. fumigatus* sens. str.,[8] while in cats, infections with cryptic species in section *Fumigati* including *A. felis*,

A. udagawae, and *A. thermomutatus* are most prevalent.[9] We performed environmental sampling for *Aspergillus* section *Fumigati* in homes of cats previously diagnosed with aspergillosis and in a recreational nature reserve and found that cryptic species reported to cause disease in humans or animals were common, comprising 25% of isolates overall.[10]

Aspergillus fumigatus sens. str. has been most commonly associated with avian infections based on phenotypic identification techniques, with *A. flavus, A. niger, A. glaucus,* and *A. nidulans* also reported.[2] Thus, cryptic species may have been overlooked as a cause of avian aspergillosis.

This study aimed to definitively identify fungal pathogens causing avian aspergillosis and to determine the prevalence of cryptic *A.* section *Fumigati* species. A secondary aim was to determine the form of aspergillosis present.

Methods

Australian avian veterinarians were contacted to recruit isolates from clinical cases of avian aspergillosis between 2013 and 2017. *Aspergillus i*solates cultured from lesions in captive birds consistent with aspergillosis, including fungal plaques or granulomas observed within the respiratory tract or other parenchyma during diagnostic investigation or at post mortem were included for study. Morphological identification of *Aspergillus* species was performed by microbiologists, based on consistent macroscopic and microscopic features including identification of smooth or rough walled conidia stemming from radiate or columnar conidial heads with globose or subclavate vesicles, stipe and a foot cell.[11] Thirty isolates were received from Australian native and exotic avian species (Table 1), including 26 from zoos (New South Wales [$n = 23$, Taronga Conservation Society approved opportunistic sampling request R14B178], South Australia [$n = 3$]), one from private practice (Queensland) and three from commercial poultry producers (NSW). Histopathology post-mortem reports were available for review in 20 zoological cases.

All isolates were subcultured onto malt extract agar and grown in the dark at 25° C for up to 7 days until sporulation occurred. DNA was extracted using the MoBio DNA isolation kit (QIAGEN MO BIO, Carlsbad, USA). Conventional polymerase chain reaction (PCR) was performed targeting the rDNA gene cluster, including the ITS1, 5.8S gene, and ITS2 regions using primers ITS1 and ITS4.[12] Amplification of the partial β-tubulin (BenA) gene was performed using primers bt2a and bt2b.[13] PCR product was sent to an external laboratory for purification and Sanger sequencing (Macrogen, Seoul, South Korea). Generated sequences were edited using BioEdit version 7.2.5.[14] Species identification was determined using NCBI-BLAST tool and sequence homology of ≥99% (ITS) and ≥99.5% (BenA) with type strains sequences.[15] If BenA homology was <99.5%, a separate alignment and phylogenetic analysis was performed in Mega 6.06[16] with sequences of all section type-strains to confirm the closest type-strain match using the maximum likelihood method based on the Tamura-Nei model with gamma distribution and 1000 bootstrap replicates. Sequences were deposited in GenBank (MF540250-MF540309).

Results

Twenty-six of 30 (87%) isolates were identified as *A. fumigatus* sens. str., and the remaining four (13%) were other *Aspergillus* species, including *A. restrictus* ($n = 1$), *A. flavus* sens. str. ($n = 2$), and *A. nidulans*-clade ($n = 1$) (Table 1). The latter isolate had 99.1% BenA homology with *A. nidulans* sens. str. Phylogenetic analysis with section *Nidulantes* type-strains placed this isolate in the *A. nidulans*-clade, where it was most closely related to *A. pachychristatus* and *A. latilabiatus*.

Affected avian species were 70% native and 30% exotic to Australia; passerines were most commonly affected (30%). Five *A. fumigatus* sens. str. isolates were from IUCN listed critically endangered native species (http://www.iucnredlist.org/search) including Plains Wanderer (*Pedionomus torquatus*) (3), Regent Honeyeater (*Anthochaera Phrygia*) (1), and Swift Parrot (*Lathamus discolor*) (1).

Histopathology findings are summarized in supplementary Table 1. Seventeen of 20 cases had invasive aspergillosis, which was disseminated in 12 and focal in five (respiratory). There were three cases of noninvasive localized infection, involving conjunctiva, air sacs, and a coelomic cavity mass. All six respiratory cases (5 invasive, 1 noninvasive) involved the lower respiratory tract; localized to the air sacs (1), the lungs (4), or both air sacs and lungs (1). In four cases, a primary nonfungal disease process was identified including mycobacteriosis, egg yolk peritonitis, metastatic pancreatic adenocarcinoma and chronic liver disease.

Discussion

Aspergillus fumigatus sens. str. was the most common isolate amongst our study population (87%), similar to prevalence studies in humans, dogs, and the environment.[4–8] While other *Aspergillus* species were identified in 13% of cases, no cryptic section *Fumigati* species were identified. This is in contrast to prevalence studies in humans (0.75–3.7% of cases)[4–6] and reports on cats, in which invasive fungal rhinosinusitis is the most common form and is caused by cryptic species in section *Fumigati*.[9] Although our sample size was small, we found a similar prevalence for *A. flavus*

Table 1. Fungal molecular identification (internal transcribed spacer (ITS), partial beta-tubulin [BenA] gene), avian species and origin details for fungal isolates used in this study ($n = 30$), collected from 2013 to 2017. Molecular identification is based on homology percentage matches of ≥99% (ITS) and ≥99.5% (BenA) using NCBI-BLAST tool.

Fungal species	Genbank accession number (ITS; BenA)	Avian species (native/exotic)	Organ isolated from	Location
A. fumigatus sens.st.	MF540280; MF540250	Pacific Black Duck (n)	Lung	Zoo, NSW
	MF540281; MF540251	Domestic Turkey (e)	Ocular	Zoo, NSW
	MF540282; MF540252	Diamond Firetail Finch (n)	Lung	Zoo, NSW
	MF540303; MF540273	Red-browed finch (n)	Liver	Zoo, NSW
	MF540284; MF540254	Crimson Finch (n)	Lung	Zoo, NSW
	MF540283; MF540253	Metallic Starling (n)	Lung	Zoo, NSW
	MF540287; MF540257	Metallic Starling (n)	Liver	Zoo, NSW
	MF540294; MF540264	Metallic Starling (n)	Lung	Zoo, NSW
	MF540285; MF540255	Olive-backed Oriole (n)	Air sac	Zoo, NSW
	MF540288; MF540258	Red Jungle Fowl (e)	Lung	Zoo, NSW
	MF540296; MF540266	Red Jungle Fowl (e)	Abdominal mass	Zoo, NSW
	MF540289; MF540259	Little Penguin (n)	Lung	Zoo, NSW
	MF540292; MF540262	Domestic Chicken (e)	Lung	Poultry producer, NSW
	MF540293; MF540263	Rufous Owl (n)	Ocular	Zoo, NSW
	MF540295; MF540265	Regent Honeyeater (n)	Lung	Zoo, NSW
	MF540297; MF540267	Scaly-breasted Lorikeet (n)	Pericardium	Zoo, NSW
	MF540298; MF540268	Hardhead Duck (n)	Air sac	Zoo, NSW
	MF540299; MF540269	Blue Fronted Amazon Parrot (e)	Lung	Veterinarian, QLD
	MF540300; MF540270	Crimson Rosella (n)	Lung	Zoo, NSW
	MF540301; MF540271	Plains Wanderer (n)	Lung	Zoo, NSW
	MF540307; MF540277	Plains Wanderer (n)	Lung	Zoo, NSW
	MF540309; MF540279	Plains Wanderer (n)	Air sac	Zoo, NSW
	MF540305; MF540275	Swift Parrot (n)	Kidney	Zoo, SA
	MF540306; MF540276	Sacred Ibis (n)	Lung	Zoo, NSW
	MF540308; MF540278	Mandarin Duck (e)	Air sac	Zoo, SA
	MF540302; MF540272	Black-faced Cockatoo (n)	Air sac	Zoo, NSW
A. restrictus	MF540286; MF540256	Java Finch (e)	Lung	Zoo, NSW
A. flavus sens. str.	MF540290; MF540260	Domestic Chicken (e)	Lung	Poultry producer, NSW
	MF540291; MF540261	Domestic Chicken (e)	Lung	Poultry producer, NSW
A. nidulans-clade	MF540304; MF540274	Palm Cockatoo (n)	Tracheal mass	Zoo, SA

e, exotic; n, native.

sens. str. (6%) and *A. nidulans*-clade (3%) as is reported in humans (*A. flavus* 8–13%; *A. nidulans* 0.5–2.5%).[4–6]

Our study is the first to our knowledge to report aspergillosis in birds due to *A. restrictus*. This case was in a Java Finch (*Lonchura oryzivora*) with chronic liver disease and disseminated invasive aspergillosis affecting air sacs, lung, and gizzard. *Aspergillus restrictus* has also been isolated from humans with disseminated infection following a heart valve replacement, although only phenotypic identification was performed.[17,18] Our findings support an opportunistic pathogen role for this fungal species.

This study demonstrates the value of molecular characterization of avian fungal isolates. *Aspergillus* species other than *A. fumigatus* sens. str. should also be considered as potential agents of this disease and may have important implications for patient management as seen in humans and other animals. Different *Aspergillus* species isolated from birds have been shown to have different susceptibilities to commonly used antifungal therapies, including itraconazole and amphotericin B.[19] This information could prove invaluable in management of a disease that is already challenged by lack of knowledge in avian pharmacokinetics and late-stage clinical presentation.[2]

We investigated avian aspergillosis amongst captive birds including several critically endangered native Australian species. The pathogenesis of avian disease has been associated with an increased environmental load of filamentous fungi in captive/rehabilitation settings (5.7% of fungal isolates grown) compared to natural avian habitats (0.03%),[20] with an *A. fumigatus* average air count nine times greater than natural habitats reported from rehabilitation centres.[21] Furthermore, genotyping of clinical and

environmental isolates has demonstrated that the rehabilitation setting is a source of clinical disease in captive birds, likely due to this increased fungal burden and the presence of existing disease and/or stress.[21] This highlights the importance of environmental control strategies to reduce the risk of aspergillosis in captivity, and the importance of fungal load monitoring.

Overall, the diversity of *Aspergillus* genus species causing infection in captive Australian birds was limited. Surveillance of wild birds may also be important for both human and animal health, as wild animal populations can be sentinels for infectious disease.[22]

Supplementary material

Supplementary data are available at *MMYCOL* online.

Acknowledgments

The authors wish to thank veterinary pathologists Dr Lydia Tong, Dr Karrie Rose, and Dr Cheryl Sangster at Taronga Conservation Society, Mosman, New South Wales for performing histopathology, and veterinary clinicians Dr David McLelland and Dr Jenny McLelland at Adelaide Zoo, Adelaide, South Australia, and Dr Adrian Gallagher, Brisbane Bird Vet, Chermside, Queensland for providing samples.

Declaration of interest.

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

1. Neumann. Aspergillosis in domesticated birds. *J Comp Pathol*. 2016; **155**: 102–104.
2. Beernaert LA, Pasmans F, Van Waeyenberghe L, Haesebrouck F, Martel A. Aspergillus infections in birds: a review. *Avian Pathol*. 2010; **39**: 325–331.
3. Dyce KM, Sack WO, Wensing CJG, eds. *Textbook of Veterinary Anatomy*, 3rd edn. Philadelphia: Saunders, 2002.
4. Negri CE, Gonçalves SS, Xafranski H et al. Cryptic and rare *Aspergillus* species in Brazil: prevalence in clinical samples and in vitro susceptibility to triazoles. *J Clin Microbiol*. 2014; **52**: 3633–3640.
5. Balajee SA, Kano R, Baddley JW et al. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *J Clin Microbiol*. 2009; **47**: 3138–3141.
6. Alastruey-Izquierdo A, Mellado E, Peláez T et al. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrob Agents Chemother*. 2013; **57**: 3380–3387.
7. Sabino R, Veríssimo C, Parada H et al. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. *Med Mycol*. 2014; **52**: 519–529.
8. Talbot JJ, Johnson LR, Martin P et al. What causes canine sino-nasal aspergillosis? A molecular approach to species identification. *Vet J*. 2014; **200**: 17–21.
9. Barrs VR, van Doorn TM, Houbraken J et al. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One*. 2013; **8**: e64871.
10. Talbot JJ, Houbraken J, Frisvad JC et al. Discovery of *Aspergillus frankstonensis* sp. nov. during environmental sampling for animal and human fungal pathogens. *PLoS One*. 2017; **12**: e0181660.
11. Samson RA, Houbraken J, Thrane U, Frisvad JC (eds). *Food and Indoor Fungi*. Utrecht: Centraalbureau voor Schimmelcultures, 2010.
12. White TJ, Bruns T, Lee SJWT. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols. A Guide to Methods and Applications*. New York: Academic Press, 1990: 315–322.
13. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol*. 1995; **61**: 1323–1330.
14. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids*. 1999; **41**: 95–98.
15. Raja HA, Miller AN, Pearce CJ, Oberlies NH. Fungal identification using molecular tools: a primer for the natural products research community. *J Nat Prod*. 2017; **80**: 756–770.
16. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*. 2013; **30**: 2725–2729.
17. Pospísil K, Straka V, Otcenásek M et al. Mycotic ulcerative aortitis after replacement of the aortic valve caused by the fungus *Aspergillus restrictus*. *Vnitř Lék*. 1984; **30**: 292–297.
18. Mencl K, Otčenàšek M, Špaček J, Řehulová E. *Aspergillus restrictus* and *Candida parapsilosis* - agents of endocarditis after heart valve replacements. *Mykosen*. 1985; **28**: 127–133.
19. Silvanose CD, Bailey TA, Di Somma A. Susceptibility of fungi isolated from the respiratory tract of falcons to amphotericin B, itraconazole and voriconazole. *Vet Rec*. 2006; **159**: 282–284.
20. Burco JD, Massey G, Byrne BA et al. Monitoring of fungal loads in seabird rehabilitation centers with comparisons to natural seabird environments in Northern California. *J Zoo Wildl Med*. 2014; **45**: 29–40.
21. Burco JD, Etienne KA, Massey JG, Ziccardi MH, Balajee SA. Molecular sub-typing suggests that the environment of rehabilitation centers may be a potential source of *Aspergillus fumigatus* infecting rehabilitating seabirds. *Med Mycol*. 2012; **50**: 91–98.
22. Reif JS. Animal sentinels for environmental and public health. *Public Health Rep*. 2011; **126**: 50–57.

Appendix 4: Azole resistance in canine and feline isolates of *Aspergillus fumigatus*

Comparative Immunology, Microbiology and Infectious Diseases 42 (2015) 37–41

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Azole resistance in canine and feline isolates of *Aspergillus fumigatus*

Jessica J. Talbot[a], Sarah E. Kidd[b], Patricia Martin[c], Julia A. Beatty[a], Vanessa R. Barrs[a,*]

[a] Valentine Charlton Cat Centre, Faculty of Veterinary Science, University of Sydney, Camperdown 2006, NSW, Australia
[b] National Mycology Reference Centre, Microbiology and Infectious Diseases, SA Pathology, Frome Road, Adelaide 5000, SA, Australia
[c] Veterinary Pathology Diagnostic Services, Faculty of Veterinary Science, University of Sydney, Camperdown 2006, NSW, Australia

ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form 13 August 2015
Accepted 18 August 2015

Keywords:
Aspergillus fumigatus
Aspergillosis
Azole resistance
Enilconazole
Clotrimazole

ABSTRACT

Azole resistance is an emerging cause of treatment failure in humans with aspergillosis. The aim of this study was to determine if azole resistance is emerging in *Aspergillus fumigatus* isolates from canine and feline sino-nasal aspergillosis cases. Susceptibilities of isolates collected between 1988 and 2014 from 46 dogs and 4 cats to itraconazole, posaconazole, voriconazole, fluconazole and ketoconazole were assessed using Sensititre YeastOne microdilution trays; and to enilconazole and clotrimazole, following the CLSI M38-A2 standard. For the majority of isolates MICs were high for ketoconazole, low for enilconazole and clotrimazole, and less than established epidemiological cut-off values for itraconazole, posaconazole and voriconazole. One canine isolate from 1992 had multiazole resistance and on *Cyp51A* gene sequencing a mutation associated with azole resistance (F46Y) was detected. There is no evidence of emerging azole resistance among *A. fumigatus* isolates from dogs and cats and topical azole therapy should be effective against most isolates.

1. Introduction

Azole antifungal drugs are pillars of treatment for aspergillosis in humans and animals. Since 1997, there has been an increasing number of reports of azole resistance amongst isolates of *Aspergillus fumigatus* from human patients with both invasive and non-invasive forms of aspergillosis. Azole resistance is associated with treatment failures and increased mortality [1–3]. Resistance to itraconazole alone or multiazole or panazole resistance has been reported globally [4–7].

Fungal azole resistance can be intrinsic or acquired. Intrinsic resistance is reported for cryptic *Aspergillus* species, whereas acquired resistance is increasingly described for *A. fumigatus sensu stricto* isolates [8,9]. Resistant strains have been identified in the environment and in human patients naïve to and previously exposed to azole therapy [10]. Resistance is associated with mutations in the *cyp51A* gene which encodes lanosterol 14α-demethylase, a component of the ergosterol synthesis pathway targeted by triazole drugs [9,11]. Emergence of resistance in Europe has been linked to triazole fungicide use in agriculture causing altered gene expression [12].

Clinical antifungal resistance is defined as persistence of infection despite treatment with an antifungal with proven *in vitro* activity against the infecting fungus [13]. *In vitro* resistance is difficult to define as although standardised methods of antifungal susceptibility testing have been developed [14–16], clinical breakpoints based on minimum inhibitory concentrations (MICs), pharmacological parameters, animal data and clinical outcomes, have not yet been established for *Aspergillus* species. Instead, epidemiological cut-off values (ECVs) of wild-type *A. fumigatus* isolates are used to evaluate clinical isolate minimum inhibitory concentrations (MICs). Correlation between *in vitro* and clinical outcomes is reported from invasive and non-invasive human aspergillosis cases [1–3].

Canine and feline sino-nasal aspergillosis (SNA) is most commonly caused by members of *Aspergillus* section *Fumigati*. Canine SNA is usually caused by *A. fumigatus* [17] while feline SNA is most commonly caused by *A. fumigatus* and *Aspergillus niger* [18]. These infections present therapeutic challenges for veterinarians, and involve the use of endosurgical procedures and topical and/or systemic antifungal azole administration. Topical clotrimazole (CLO) or enilconazole (ENL) are the mainstays of therapy, although there are limited *in vitro* studies to support the use of these drugs [19,20].

Abbreviations: SNA, sino-nasal aspergillosis; SOA, sino-orbital aspergillosis; ITZ, itraconazole; POS, posaconazole; FLU, fluconazole; KET, ketoconazole; VOR, voriconazole; AMB, amphotericin B; CAS, caspofungin; 5-FC, 5-flucytosine; CLO, clotrimazole; ENL, enilconaozle; MIC, minimum inhibitory concentration; MEC, minimum effective concentration.

* Corresponding author. Tel.: +61 2 9351 3437; fax: +61 2 9351 4261.
E-mail address: vanessa.barrs@sydney.edu.au (V.R. Barrs).

http://dx.doi.org/10.1016/j.cimid.2015.08.002
0147-9571/

Response rates to treatment vary [18,21,22]. Whether emerging azole resistance contributes to treatment failures is unknown. To date no studies have systematically investigated the prevalence of azole resistance amongst *A. fumigatus* isolates from dogs and cats. To determine whether azole resistance is emerging amongst *A. fumigatus* isolates from dogs and cats susceptibilities to a panel of antifungal drugs were assessed in a retrospective surveillance study.

2. Materials and methods

2.1. Isolates

Fifty archived isolates of *A. fumigatus* from dogs ($n=46$) and cats ($n=4$) with confirmed SNA were retrieved from the fungal culture biobank of the Small Animal Infectious Diseases Clinical Research Group at the University of Sydney [17,21,23]. Isolates originated from Australia ($n=33$), United States of America ($n=10$) and Belgium ($n=7$). Collection dates ranged from 1988 to 2013 inclusive [1988 ($n=1$); 1992 ($n=1$); 1994 ($n=1$); 2000 ($n=1$); 2004 ($n=1$); 2005 ($n=1$); 2006 ($n=1$); 2007 ($n=5$); 2008 ($n=7$); 2009 ($n=5$); 2010 ($n=6$); 2011 ($n=3$); 2012 ($n=14$); 2013 ($n=3$)].

2.2. Antifungal susceptibility testing

Isolates were sub-cultured onto malt extract agar and grown in the dark at 25 °C until sporulation occurred (7–14 days), then inoculum was prepared according to the Clinical Laboratory Standards Institute (CLSI) M38-A2 standard for filamentous fungi [16].

Susceptibility ($n=50$) to ENL and CLO (BOVA Compounding Chemist, St Marys, Australia) was assessed following the recommendations for drug preparation, dilution, inoculum preparation, incubation and endpoint reading stipulated by the CLSI M38-A2 standard [16]. Final concentrations tested were two fold dilutions within the range 0.03–16 μg/mL for both compounds. The concentrations selected were based on *in vitro* susceptibilities of *A. fumigatus* to other azole drugs [16,24,25] and are lower than concentrations of 1–2% (10–20 mg/mL) used in topical preparations of clotrimazole and enilconazole for treatment of canine and feline SNA [19–21,24,25]. Quality control strains used as a standard to assess repeatability and accuracy of testing procedures were reference strains of *Candida parapsilosis* (ATCC 22019) and *A. fumigatus* (ATCC MYA-3626), according to the CLSI M38-A2 standard.

Susceptibility to amphotericin B (AMB), itraconazole (ITZ), ketoconazole (KCZ), voriconazole (VCZ) (range 0.008–16 μg/mL for all four compounds), posaconazole (POS) (range 0.008–8 μg/mL), fluconazole (FLU) (0.12–256 μg/mL) and 5-flucytosine (5-FC) (0.03–64 μg/mL) was assessed using Sensititre YeastOne YO8 microdilution trays according to the manufacturer's instructions. Susceptibility to caspofungin (CSP) (range 0.008–16 μg/mL) was assessed using the CLSI M38-A2 standard [16]. For those drugs for which pharmacokinetic data are available in dogs and/or cats including itraconazole, fluconazole, ketoconazole and posaconazole, reported C_{MAX} in serum or plasma after therapeutic dosing were within the range of drug concentrations tested in this study [26–31]. *C. parapsilosis* (ATCC 22019) was used as the quality control strain (Trek Diagnostic Systems, Thermo Fisher Scientific, Scoresby, Australia).

For all isolates and both tray types, 10 μL from the growth control well was spread onto a Sabouraud's agar plate immediately after plate inoculation as a purity and inoculum density check. All trays were incubated at 35 °C for 72 h ± 2 h in an ambient air incubator. Endpoints for all drugs were determined after 72 h incubation. For CLO and ENL the endpoint was the lowest two-fold dilution revealing 100% growth inhibition (MIC). For AMB, ITZ, POS, VCZ, FLU, KCZ and 5-FC, the endpoints were as per the manufacturer's instructions (Trek Diagnostic Systems, Thermo Fisher Scientific, Scoresby, Australia). For CSP endpoints were read as MECs at the lowest dilution of round compact hyphal forms as per CLSI M38A2 [16]. Geometric mean, MIC_{50} and MIC_{90} values were calculated using Windows Excel (Microsoft Office, 2007, Redmond, United States of America).

In vitro azole resistance was defined as the absence of endpoint antifungal activity in the range of drug concentrations tested, except for FLU and 5-FC, as most filamentous fungi have intrinsic resistance to these drugs [16]. Multiazole resistance was defined as resistance to more than one antifungal azole. Decreased susceptibility was defined as MICs greater than established ECVs for wild-type *A. fumigatus*, established using CLSI standards [16].

2.3. Cyp51A gene sequencing

DNA was extracted from those isolates with possible ITZ resistance (*i.e.* MICs > 1 μg/mL) using the Roche High Pure PCR Template Preparation Kit (Castle Hill, Australia), with an additional bead beating step [32]. The coding region of *cyp51A* was sequenced as described previously [11]. Sequences were aligned with *cyp51A A. fumigatus* reference strain, GenBank reference AF338659 [33] and sequence from a reference laboratory isolate with a L98H *cyp51A* resistance mutation (obtained from National Mycology Reference Centre, SA Pathology, Adelaide, South Australia, 2014).

3. Results

Antifungal susceptibility results for all 50 *A. fumigatus* isolates from clinical specimens from dogs and cats with sino-nasal aspergillosis are listed in Table 1 and compared to established

Table 1
Antifungal susceptibility results for 50 *A. fumigatus* isolates from clinical specimens from dogs and cats and comparison with epidemiological cut-off values.* MIC/MEC (μg/mL) values reflect the number of isolates within the specific cut-off value.

Drug	MIC/MEC (μg/mL) distribution among tested isolates												GM	MIC_{50}	MIC90	ECV (μg/mL)
	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	>16				
AMB						1	8	34	7				1.92	2	4	2
ITZ		2	13	16	17	1						1	0.13	0.12	0.25	1
VCZ			2	3	24	16	3	1	1				0.34	0.25	0.5	1
POS	5	13	20	8	3	1							0.06	0.06	0.12	0.5
KCZ							1	2	28	15	3	1	5.09	4	8	–
CSP†	16	25	8	1									0.03	0.03	0.06	1
CLO					2	31	15	1	1				0.64	0.5	1	–
ENL			7	27	11	4		1					0.15	0.12	0.25	–

* ECV, epidemiological cut off value [46–48]; MIC, minimum inhibitory concentration; MEC, minimum effective concentration (†); AMB, amphotericin-B; ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; KCZ, ketoconazole; CSP, caspofungin; CLO, clotrimazole; ENL, enilconazole; GM, geometric mean; MIC_{50}, minimum inhibitory concentration at which ≥50% of isolates in the group were inhibited; MIC_{90}, minimum inhibitory concentration at which ≥90% of isolates in the group were inhibited.

Table 2
Antifungal susceptibility results for an Australian canine clinical isolate of *A. fumigatus* from 1992 compared to group GM, MIC_{50} and MIC_{90} results*.

Drug	MIC/MEC (μg/mL)	GM	MIC_{50}	MIC_{90}	ECV (μg/mL)
AMB	1	1.97	2	4	2
ITZ	>16	0.14	0.12	0.25	1
VCZ	4	0.37	0.25	0.5	1
POS	0.5	0.06	0.06	0.12	0.5
KCZ	>16	5.52	4	8	–
CSP[†]	0.03	0.03	0.06	0.03	1
CLO	4	0.39	0.5	0.5	–
ENL	2	0.15	0.125	0.5	–

* ECV, epidemiological cut-off values from [46–48]; MIC, minimum inhibitory concentration; MEC, minimum effective concentration ([†]); AMB, amphotericin-B; ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; KCZ, ketoconazole; CSP, caspofungin; CLO, clotrimazole; ENL, enilconazole.

epidemiological cut-off values (ECVs) where these have been established. All isolates had high MICs for KCZ. As expected, there was no observed activity of FLU or 5-FC against any isolate. Forty-nine of the 50 isolates had low MICs for ITZ, VCZ, POS and low MECs for CSP. Seven isolates had MICs higher than the ECV for AMB. The *C. parapsilosis* (ATCC 22019) quality control strain had MIC values within the manufacturer's defined range defined within the YeastOne YO8 product insert.

The majority of *A. fumigatus* isolates had low MICs for ENL and CLO. The *A. fumigatus* (ATCC MYA-3626) quality control strain had a MIC value of 1 μg/mL for CLO and 0.25 μg/mL for ENL. The *C. parapsilosis* (ATCC 22019) quality control strain had a MIC value of 0.125 μg/mL for CLO and 0.25 μg/mL for ENL.

There was no activity of ITZ and KCZ against one Australian canine *A. fumigatus* isolate collected in 1992. The same isolate also showed high MICs of VCZ and POS (Table 2). *Cyp51A* sequencing revealed non-synonymous mutations at nucleotide positions 137 (F46Y), 267 (G89E), 1279 (E427K) and a synonymous mutation at nucleotide 1362 (C454).

4. Discussion

In this study azole resistance was rare amongst *A. fumigatus* isolates from dogs and cats. ITZ resistance in *Aspergillus* species was first reported in 1997 from Californian isolates sampled from human patients in the late 1980s [34]. Since the late 1990s, there has been an increase in the number of reports of and surveillance programs for azole resistance in human *A. fumigatus* isolates [35]. Azole resistance in environmental and human clinical isolates has been reported in Europe, Asia, North and South America, Australia and the Middle East [2,4,5,7,36]. Emergence of resistance in Europe has been linked to triazole fungicide use on crops causing altered gene expression and this has prompted a number of surveillance programs that have continued to identify azole resistance in the region, predominantly amongst isolates of *A. fumigatus* [10,12].

The only isolate in this study to demonstrate *in vitro* azole resistance was collected from a dog in Australia in 1992. This isolate exhibited multiazole resistance, with no observed activity of ITZ/KCZ in the range tested (MIC > 16 μg/mL) (Table 2). Itraconazole MIC values >8 μg/mL in *A. fumigatus* isolates from humans with invasive aspergillosis have been associated with clinical resistance [14,34]. Correlations between *in vitro* and clinical activity of KCZ and *A. fumigatus* isolates from humans have also been reported [37]. A limitation of the current study was the lack of detailed treatment histories for affected dogs and cats.

This is the first study to systematically confirm *in vitro* susceptibility of canine and feline isolates of *A. fumigatus* to ENL and CLO using standardized methods. Pharmaceutical company MIC values for ENL against 77 *Aspergillus* spp. canine isolates were reported by a pharmaceutical company previously and were ≤1 μg/mL in 62% of these (range ≤0.1–10 μg/mL) [19]. However, isolates were not identified to species level, and the method for antifungal susceptibility testing was not described. Another study on the use of ENL to treat canine SNA reported a MIC value for *A. fumigatus* to ENL of 1 μg/mL [20]. However, the number of isolates tested and the method of testing was not reported.

We found that canine and feline isolates of *A. fumigatus* are susceptible to ENL and CLO. *A. fumigatus* isolates had MIC values for CLO between 0.25 and 4 μg/mL and for ENL between 0.06 and 2 μg/mL. Therefore, assuming that *in vitro* MICs correlate with clinical response, currently used concentrations (10 mg/mL) of topical azole therapy should be effective against *A. fumigatus* isolates causing SNA in dogs and cats, and azole resistance is unlikely to be a cause of treatment failure at this time. One percent solutions of CLO and ENL are standard for the treatment of canine SNA, with approximately 50 mL of 10 mg/mL solution infused into the sinonasal cavities of large breed dogs to ensure that high concentrations of the drug contact fungal plaques [24,25]. Additional treatment with at least 20 g of CLO 10 mg/g cream has also been used following infusion therapy [24]. CLO infusions have also been used in cats with SNA [18].

A recent study by the authors to determine the molecular identification of canine SNA isolates confirmed the most common aetiological agent as *A. fumigatus* (88/91 isolates), thus different aetiological agents are not the cause of the varied clinical response to treatment reported in dogs [17]. CLO and ENL were found to have *in vitro* activity against all isolates in this study. A likely impediment to disease resolution is the inability of azoles to adequately penetrate into mucosal fungal plaques. Endoscopic debridement of all visible plaques is considered essential to achieve disease resolution in both dogs and cats with SNA [18,38]. In human fungal rhinosinusitis due to *A. fumigatus*, fungal balls are endosurgically removed and usually no topical antifungal therapy is applied or required to achieve therapeutic success [39,40]. Another possible cause of treatment failure in canine and feline SNA is a lack of adequate drug distribution to the affected areas of the nasal cavity and frontal sinus when applying topical preparations of azoles [38,41,42].

Topical azole therapy is contraindicated in the presence of cribriform plate breaches in dogs and cats with SNA due to the potential for chemical meningoencephalitis. In these cases systemic antifungal therapy is offered in addition to topical fungal plaque debridement. We recommend that *in vitro* antifungal susceptibility testing be performed to determine resistant phenotypes. The seven isolates with high MICs for AMB (>2 μg/mL) in this study highlight the importance of performing *in vitro* susceptibility in cases where systemic therapy is being considered. In human studies, MIC values >2 μg/mL have been associated with treatment failure, while values <2 μg/mL have been associated with treatment success in patients with invasive *A. fumigatus* infections [43]. The significance of these MIC values has not yet been determined dogs and cats with sino-nasal aspergillosis.

High KCZ MIC results (≥MIC_{90} 8 μg/mL) were present in 38% (19/50) of *A. fumigatus* isolates in this study, suggesting that KCZ is unsuitable for treatment of canine or feline SNA. These MICs are

similar to those reported for human clinical *A. fumigatus* isolates [44,45].

Combinations of topical azoles and systemic antifungals have also been used to treat canine and feline SNA. Previously KCZ and topical ENL were used for canine SNA treatment [19]. More recently combination therapy with oral ITZ and topical therapy has been employed [46]. Feline SNA has also been treated with a combination of systemic and topical intranasal infusions or topical intranasal infusions only. Therapeutic success of systemic (ITZ or POS ± AMB), topical (CLO) and combined antifungal therapies has been reported in 14 cases of feline SNA, with a success rate of 79% [18].

Epidemiological cut-off values (ECVs) for wild type *A. fumigatus* isolates to AMB, ITZ, POS, VCZ and CSP have been established using CLSI standards for broth microdilution testing. These ECVs may be useful for identifying resistant phenotypes with non-wild type MICs. For AMB 3988 wild type *A. fumigatus* isolates were tested and 95% had MICs of 2 μg/mL [47]. In the current study, 86% ($n = 43$) of the *A. fumigatus* isolates were found to have MICs ≤ 2 μg/mL. The remaining 14% of isolates ($n = 7$) had higher MIC values (4 μg/mL). Of 1684–2815 wild type, *A. fumigatus* isolates tested for susceptibility to ITZ, POS and VCZ, 98.8% had MIC values of 1 μg/mL for ITZ; 99.2% had MICs of 0.5 μg/mL for POS; and 97.7% had MICs of 1 μg/mL for VCZ [48]. In the current study all isolates had MICs for POS ≤ 0.5 μg/mL and only one isolate had ITZ MIC > 1 μg/mL (16 μg/mL). The same isolate and one USA isolate from 2008 had VCZ MICs > 1 μg/mL (4 μg/mL and 2 μg/mL, respectively). Of 1691 wild type *A. fumigatus* isolates tested for susceptibility to CSP, 99% had MECs of 1 μg/mL [49]. In the current study all isolates had MECs for CSP <1 μg/mL. These results imply that compared to the ECVs for wild type *A. fumigatus* isolates, resistant phenotypes were rare amongst this cohort of isolates, although resistance for AMB ($n = 7$), ITZ ($n = 1$) and VCZ ($n = 2$) was observed.

The most common *cyp51A* mutations associated with azole resistance in *A. fumigatus* are a SNP (L98H) in combination with a 34 bp tandem repeat in the gene promoter region (TR_{34}/L98H) or a 46 bp tandem repeat in the same region and two separate SNPs (TR_{46}/Y156F/T289A) [4]. In this study the *cyp51A* amino acid substitutions found in the 1992 azole resistant canine isolate mostly occurred in regions outside the coding region and are therefore unlikely to contribute to resistance [50,51]. However, the F46Y mutation we detected, has been previously associated with reduced susceptiblity to triazoles [7]. Azole resistant isolates lacking mutations in the *cyp51A* gene have been previously reported [50]. Other proposed resistance mechanisms include upregulation of efflux pumps, expression levels of transporters and multidrug resistance proteins or other undiscovered mechanisms [50].

5. Conclusions

This study determined that azole resistance is not emerging in *A. fumigatus* isolates from dogs and cats with SNA. In general, MICs were similar to those reported for wild type isolates for ITZ, POS, VCZ, AMB and CSP, implying that canine and feline isolates of *A. fumigatus* are generally susceptible to these antifungals. MICs of *A. fumigatus* isolates to ENL and CLO were relatively low and concentrations currently used for topical therapy should be effective. Further studies would be useful to investigate *in vitro* versus clinical outcomes in a greater number of cases to help develop interpretive guidelines for antifungal susceptibility testing.

Acknowledgements

The authors thank Jan Bell, SA Pathology at the Women's and Children's Hospital, North Adelaide, for technical assistance.

References

[1] P.R. Burgel, M.T. Baixench, M. Amsellem, et al., High prevalence of azole-resistant *Aspergillus fumigatus* in adults with cystic fibrosis exposed to itraconazole, Antimicrob. Agents. Chemother. 56 (2011) 869–874.
[2] I. Hadrich, F. Makni, S. Neji, et al., Amphotericin B *in vitro* resistance is associated with fatal *Aspergillus flavus* infection, Med. Mycol. 50 (2012) 829–834.
[3] J.W. van der Linden, E. Snelders, G.A. Kampinga, et al., Clinical implications of azole-resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009, Emerg. Infect. Dis. 17 (2011) 1846–1854.
[4] D.W. Denning, D.S. Perlin, Azole resistance in *Aspergillus*: a growing public health menace, Future Microbiol. 6 (2011) 1229–1232.
[5] C.D. Pham, E. Reiss, F. Hagen, et al., Passive surveillance for azole-resistant *Aspergillus fumigatus*, United States, 2011–2013, Emerg. Infect. Dis. 20 (2014) 1498–1503.
[6] P.E. Verweij, S.J. Howard, W.J. Melchers, et al., Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints, Drug Resist. Updat. 12 (2009) 141–147.
[7] S.E. Kidd, E. Goeman, J.F. Meis, et al., Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia, Mycoses 58 (2015) 350–355.
[8] S.J. Howard, Multi-resistant aspergillosis due to cryptic species, Mycopathologia 178 (2014) 435–439.
[9] J.W. van der Linden, A. Warris, P.E. Verweij, *Aspergillus* species intrinsically resistant to antifungal agents, Med. Mycol. 49 (S1) (2011) S82–S89.
[10] S.J. Howard, M.C. Arendrup, Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection, Med. Mycol. 49 (S1) (2011) S90–S95.
[11] J. Chen, H. Li, R. Li, et al., Mutations in the cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma, Antimicrob. Agents. Chemother. 55 (2005) 31–37.
[12] P.E. Verweij, G.H. Kema, B. Zwaan, et al., Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould *Aspergillus fumigatus*, Pest. Manage. Sci. 69 (2012) 165–170.
[13] Z.A. Kanafani, J.R. Perfect, Resistance to antifungal agents: mechanisms and clinical impact, Antimicrob. Resist. 46 (2008) 120–128.
[14] D.W. Denning, S.A. Radford, K.L. Oakley, et al., Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatus* infection, J. Antimicrob. Chemother. 40 (1997) 401–414.
[15] C. Lass-Flörl, M. Cuenca-Estrella, D.W. Denning, Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology, Med. Mycol. 44 (2006) S319–S325.
[16] J.H. Rex, B.D. Alexander, D. Andes, et al., Reference method for broth dilution antifungal susceptibility testing of filamentous fungi Approved Standard M38-A2, vol. 28, second ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2008, pp. 1–35 (no. 16).
[17] J.J. Talbot, P. Martin, L. Johnson, et al., What causes sino-nasal aspergillosis in dogs? A molecular approach to species identification, Vet. J. 200 (2014) 17–21.
[18] V.R. Barrs, J.J. Talbot, Feline aspergillosis, Vet. Clin. North Am. Small Anim. Pract. 44 (2014) 51–73.
[19] N.J.H. Sharp, M. Sullivan, C.E. Harvey, et al., Treatment of canine nasal aspergillosis with enilconazole, J. Vet. Intern. Med. 7 (1993) 40–43.
[20] T. Lanthier, A. Chalifoux, Enilconazole as an adjunct to the treatment of 4 cases of canine nasal aspergillosis, Can. Vet. J. 32 (1991) 110–112.
[21] V.R. Barrs, C. Halliday, P. Martin, et al., Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes, Vet. J. 191 (2012) 58–64.
[22] K.G. Mathews, N.J.H. Sharp, Canine nasal aspergillosis-penicilliosis, in: C.E. Green (Ed.), Infectious Diseases of the Dog and Cat, third ed., Saunders Elsevier, St Louis, MO, 2006, pp. 613–620.
[23] V.R. Barrs, T.M. van Doorn, J. Houbraken, et al., *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs, PLoS ONE 8 (2013), DOI 64810.61371/journal.pone.0064871.
[24] T.R. Sissener, N.J. Bacon, E. Friend, et al., Combined clotrimazole irrigation and depot therapy for canine nasal aspergillosis, J. Small Anim. Pract. 47 (2006) 312–315.
[25] J.L. Zonderland, C.K. Stork, J.H. Saunders, et al., Intranasal infusion of enilconazole for treatment of sinonasal aspergillosis in dogs, J. Am. Vet. Med. Assoc. 221 (2002) 1421–1425.
[26] D.M. Boothe, I. Herring, J. Calvin, et al., Itraconazole disposition after single oral and intravenous and multiple oral dosing in healthy cats, Am. J. Vet. Res. 58 (1997) 872–877.
[27] H. Van Cauteren, J. Heykants, R. De Coster, et al., Itraconazole: pharmacologic studies in animals and humans, Rev. Infect. Dis. 9 (1987) S43–S46.
[28] S.L. Vaden, M.C. Heit, E.C. Hawkins, et al., Fluconazole in cats: pharmacokinetics following intravenous and oral administration and penetration into cerebrospinal fluid, aqueous humour and pulmonary epithelial lining fluid, J. Vet. Pharmacol. Ther. 20 (1997) 181–186.
[29] M.J. Humphrey, S. Jevons, M.H. Tarbit, Pharmacokinetic evaluation of UK-49,858, a metabolically stable triazole antifungal drug, in animals and humans, Antimicrob. Agents Chemother. 28 (1985) 648–653.
[30] J.G. Baxter, C. Brass, J.J. Schentag, et al., Pharmacokinetics of ketoconazole administered intravenously to dogs and orally as tablet and solution to humans and dogs, J. Pharm. Sci. 75 (1986) 443–447.
[31] A.A. Nomeir, P. Kumari, M.J. Hilbert, et al., Pharmacokinetics of SCH 56592, a new azole broad-spectrum antifungal agent, in mice, rats, rabbits, dogs, and cynomolgus monkeys, Antimicrob. Agents Chemother. 44 (2000) 727–731.

[32] P.L. White, M.D. Perry, J. Loeffler, et al., Critical stages of extracting DNA from *Aspergillus fumigatus* in whole-blood specimens, J. Clin. Microbiol. 48 (2010) 3753–3755.
[33] E. Mellado, T.M. Diaz-Guerra, M. Cuenca-Estrella, et al., Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species, J. Clin. Microbiol. 39 (2001) 2431–2438.
[34] D.W. Denning, K. Venkateswarlu, K.L. Oakley, et al., Itraconazole resistance in *Aspergillus fumigatus*, Antimicrob. Agents Chemother. 41 (1997) 1364–1368.
[35] S.P. Georgiadou, D.P. Kontoyiannis, The impact of azole resistance on aspergillosis guidelines, Ann. N.Y. Acad. Sci. 1272 (2012) 15–22.
[36] A. Chowdhary, S. Kathuria, J. Xu, et al., Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health, PLoS Pathog. 9 (2013), http://dx.doi.org/10.1371/annotation/4ffcf1da-b180-4149-834c-9c723c5dbf9b.
[37] E. Cauvenbergh, J. Symoens, Clinical experience with ketoconazole, a review, Clin. Res. Rev. 1 (1981) 195–199.
[38] M.J. Sharman, C.S. Mansfield, Sinonasal aspergillosis in dogs: a review, J. Small Anim. Pract. 53 (2012) 434–444.
[39] X. Dufour, C. Kauffman-Lacroix, J.C. Ferrie, et al., Paranasal sinus fungus ball and surgery: a review of 175 cases, Rhinology 43 (2005) 34–39.
[40] M.G. Zuniga, J.H. Turner, Treatment outcomes in acute invasive fungal rhinosinusitis, Curr. Opin. Otolaryngol. Head Neck Surg. 22 (2014) 242–248.
[41] G.M. Hayes, J.L. Demetriou, Distribution and persistence of topical clotrimazole after sinus infusion in normal canine cadavers, J. Small Anim. Pract. 53 (2012) 95–100.
[42] R. Burrow, D. McCarroll, M. Baker, et al., Frontal sinus depth at four landmarks in breeds of dog typically affected by sinonasal aspergillosis, Vet. Rec. 170 (2012), http://dx.doi.org/10.1136/vr.100041.
[43] C. Lass-Florl, G. Kofler, G. Kropshofer, et al., *In vitro* testing of susceptibility to amphotericin B is a reliable predictor of clinical outcome in invasive aspergillosis, J. Antimicrob. Chemother. 42 (1998) 497–502.
[44] A.J. Carrillo-Muñoz, G. Quindós, M. Ruesga, et al., In vitro antifungal susceptibility testing of filamentous fungi with Sensititre Yeast One, Mycoses 49 (2006) 293–297.
[45] S.A. Messer, R.N. Jones, T.R. Fritsche, International Surveillance of *Candida* spp. and *Aspergillus* spp.: report from the SENTRY Antimicrobial Surveillance Program (2003), J. Clin. Microbiol. 44 (2006) 1782–1787.
[46] S. Claeys, J.B. Lefebvre, S. Schuller, et al., Surgical treatment of canine nasal aspergillosis by rhinotomy combined with enilconazole infusion and oral itraconazole, J. Small Anim. Pract. 47 (2006) 320–324.
[47] A. Espinel-Ingroff, M. Cuenca-Estrella, A. Fothergill, et al., Wild-Type MIC distributions and epidemiological cutoff values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document), Antimicrob. Agents Chemother. 55 (2011) 5150–5154.
[48] A. Espinel-Ingroff, D.J. Diekema, A. Fothergill, et al., Wild-Type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document), J. Clin. Microbiol. 48 (2010) 3251–3257.
[49] A. Espinel-Ingroff, A. Fothergill, J. Fuller, et al., Wild-Type MIC distributions and epidemiological cutoff values for caspofungin and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document), Antimicrob. Agents Chemother. 55 (2011) 2855–2859.
[50] E. Snelders, A. Karawajczyk, G. Schaftenaar, et al., Azole resistance profile of amino acid changes in *Aspergillus fumigatus CYP51A* based on protein homology modeling, Antimicrob. Agents Chemother. 54 (2010) 2425–2430.
[51] S.J. Howard, D. Cerar, M.J. Anderson, et al., Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure, Emerg. Infect. Dis. 15 (2009) 1068–1076.

Appendix 5: Surveillance for azole resistance in clinical and environmental isolates of *Aspergillus fumigatus* in Australia and *cyp51A* homology modelling of azole-resistant isolates

This is a pre-copy edited, author-produced version of an article accepted for publication in *Medical Mycology* following peer review. The version of record **Talbot J.J.,** Subedi S., Halliday C., et al., Surveillance for azole resistance in clinical and environmental isolates of *Aspergillus fumigatus* in Australia and *cyp51A* homology modelling of azole-resistant isolates, *Journal of Antimicrobial Chemotherapy*, 2018, dky187, is available online at https://doi.org/10.1093/jac/dky187.

J Antimicrob Chemother
doi:10.1093/jac/dky187

Journal of Antimicrobial Chemotherapy

Surveillance for azole resistance in clinical and environmental isolates of *Aspergillus fumigatus* in Australia and *cyp51A* homology modelling of azole-resistant isolates

Jessica J. Talbot[1]*†, Shradha Subedi[2,3]†, Catriona L. Halliday[2], David E. Hibbs[4], Felcia Lai[4], Francisco J. Lopez-Ruiz[5], Lincoln Harper[5], Robert F. Park[6], William S. Cuddy[7], Chayanika Biswas[2,8], Louise Cooley[9], Dee Carter[8,10], Tania C. Sorrell[8], Vanessa R. Barrs[1,8]‡ and Sharon C.-A. Chen[2,8]‡

[1]*Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, New South Wales, Australia;* [2]*Centre for Infectious Diseases and Microbiology Laboratory Services, ICPMR, New South Wales Health Pathology, Westmead Hospital, The University of Sydney, Westmead, New South Wales, Australia;* [3]*Department of Infectious Diseases, Sunshine Coast University Hospital, Queensland, Australia;* [4]*Faculty of Pharmacy, The University of Sydney, New South Wales, Australia;* [5]*Centre for Crop and Disease Management, School of Molecular and Life Sciences, Curtin University, Bentley, Western Australia, Australia;* [6]*Judith & David Coffey Chair of Sustainable Agriculture, University of Sydney Plant Breeding Institute Cobbitty, The University of Sydney, New South Wales, Australia;* [7]*NSW Department of Primary Industries, co-located at the Elizabeth Macarthur Agricultural Institute, Menangle and the University of Sydney's Plant Breeding Institute Cobbitty, The University of Sydney, New South Wales, Australia;* [8]*The University of Sydney, Marie Bashir Institute for Infectious Diseases and Biosecurity and Westmead Clinical School and The Centre for Infectious Diseases and Microbiology, Westmead Institute for Medical Research, Westmead, New South Wales, Australia;* [9]*Department of Microbiology and Infectious Diseases, Royal Hobart Hospital, Hobart, Tasmania, Australia;* [10]*School of Life and Environmental Sciences, The University of Sydney, New South Wales, Australia*

*Corresponding author. Tel: +612-9351-3437; Fax: +612-9351-7436; E-mail: jessica.talbot@sydney.edu.au
†Equal contribution.
‡Equal joint senior authors.

Received 2 January 2018; returned 20 February 2018; revised 17 April 2018; accepted 19 April 2018

Background: The prevalence of azole resistance in *Aspergillus fumigatus* is uncertain in Australia. Azole exposure may select for resistance. We investigated the frequency of azole resistance in a large number of clinical and environmental isolates.

Methods: *A. fumigatus* isolates [148 human, 21 animal and 185 environmental strains from air ($n = 6$) and azole-exposed ($n = 64$) or azole-naive ($n = 115$) environments] were screened for azole resistance using the VIPcheck™ system. MICs were determined using the Sensititre™ YeastOne YO10 assay. Sequencing of the *Aspergillus cyp51A* gene and promoter region was performed for azole-resistant isolates, and *cyp51A* homology protein modelling undertaken.

Results: Non-WT MICs/MICs at the epidemiological cut-off value of one or more azoles were observed for 3/148 (2%) human isolates but not amongst animal, or environmental, isolates. All three isolates grew on at least one azole-supplemented well based on VIPcheck™ screening. For isolates 9 and 32, the itraconazole and posaconazole MICs were 1 mg/L (voriconazole MICs 0.12 mg/L); isolate 129 had itraconazole, posaconazole and voriconazole MICs of >16, 1 and 8 mg/L, respectively. Soil isolates from azole-exposed and azole-naive environments had similar geometric mean MICs of itraconazole, posaconazole and voriconazole ($P > 0.05$). A G54R mutation was identified in the isolates exhibiting itraconazole and posaconazole resistance, and the TR_{34}/L98H mutation in the pan-azole-resistant isolate. *cyp51A* modelling predicted that the G54R mutation would prevent binding of itraconazole and posaconazole to the haem complex.

Conclusions: Azole resistance is uncommon in Australian clinical and environmental *A. fumigatus* isolates; further surveillance is indicated.

Introduction

Aspergillus fumigatus causes aspergillosis in humans and animals ranging from chronic forms to acute invasive aspergillosis (IA). IA, in particular, presents significant problems for haematological patients, with mortalities of >50%.[1,2] Antifungal triazoles are first-line agents for managing these infections and preventing fungal diseases in agriculture and construction.[3] The emergence of azole-resistant strains of *A. fumigatus* (ARAf) is thus concerning, being associated with treatment failure and resistance, and may be particularly relevant in haematology patients, many of whom have prior exposure to azoles.[2,4–6] Selection pressure for ARAf has also been linked to exposure to agricultural azole-based fungicides, the demethylation inhibitors (DMIs).[5–7] Major mechanisms of azole resistance in ARAf include SNPs in the *A. fumigatus cyp51A* gene, alone or combined with tandem repeats (TRs) in the promoter region.[8–10]

The prevalence of ARAf varies with geographical region and is highest in Europe (3%–26%).[5,8,10] In Australia, while ARAf isolates have been identified,[11] their prevalence is uncertain. To better understand the burden of ARAf, we investigated the frequency of resistance to itraconazole, voriconazole and posaconazole among clinical and environmental *A. fumigatus* isolates.

Methods

Human isolates of *A. fumigatus* ($n = 148$) were obtained from culture collections at Westmead and Royal Hobart Hospitals from 2015 to 2017 (Table S1, available as Supplementary data at *JAC* Online). Of these, 35 isolates were from patients with physician-ascribed IA using established criteria where appropriate (see Table S1).[12] Twenty-one animal isolates (School of Veterinary Science, University of Sydney, 2013–17; Table S1) and 185 environmental isolates from azole-naive ($n = 115$) or azole-exposed ($n = 64$) habitats and ambient air ($n = 6$) (2013–17; Table S2) were also studied.

For isolation of *A. fumigatus*, soil samples were stored at 4°C and processed within 1 week (method 1),[13] with 100 μL of soil suspension incubated at 45°C on malt extract agar containing chloramphenicol (MEASC). Early experiments showed that for azole-treated soils, *A. fumigatus* yields were low and samples were reprocessed using 'method 2' as previously described.[14] Briefly, two sets of 5× serial 1:10 dilutions of soil were analysed. One set was heat treated at 75°C for 30 min and 100 μL from each dilution was incubated at 37°C on MEASC for 21 days. Colonies were identified as *A. fumigatus* species complex by standard morphological methods.[15] Identity was confirmed as *A. fumigatus sensu stricto* by *β-tubulin* gene sequencing.[16]

To screen for azole resistance, *Aspergillus* isolates were inoculated onto 4-well VIPcheck™ plates (Balis Laboratorium VOF, Boven Leeuwen, The Netherlands) with wells supplemented with itraconazole (4 mg/L), voriconazole (2 mg/L) or posaconazole (0.5 mg/L), or unsupplemented.[17] Isolates growing only in the control well were considered azole susceptible. Isolates that grew on any azole-containing well underwent re-examination and susceptibility testing using the Sensititre™ YeastOne YO10 system (TREK Diagnostics Systems Ltd, OH, USA).[18] Both Sensititre™ and VIPcheck™ testing was repeated for isolates that grew on azole-supplemented wells.

Susceptibility testing was performed on all human isolates and randomly selected animal ($n = 11$, 52%) and environmental ($n = 41$, 22%) isolates according to the manufacturer's instructions and following CLSI methodology (Third Edition: M38).[18] MICs or minimum effective concentrations (MECs) were read after 48 h of incubation and determined by visual inspection. *Candida parapsilosis* ATCC 22019 (GenBank reference NRRL Y-12969) was the quality control, and *A. fumigatus* ATCC 204305 (GenBank reference KC689329, KU729126 and KU897017) was the reference strain.[18] MICs or MECs were assessed using established criteria, with MICs read at 100% inhibition, i.e. no signs of visual growth.[18] Interpretative criteria based on epidemiological cut-off values (ECVs) were: for itraconazole and voriconazole, an MIC of ≤1 mg/L was WT and ≥2 mg/L was non-WT (NWT); and for posaconazole, an MIC of ≤0.25 mg/L was WT and >0.5 mg/L was NWT.[19,20] Geometric mean (GM) MICs/MECs were calculated for WT isolates. Standard deviations and *t*-scores compared GM MICs for groups of isolates with significance set at $P \leq 0.05$. For isolates with NWT MICs, the *Aspergillus cyp51A* gene and promoter region were amplified and sequenced.[21] Sequences were aligned with GenBank reference sequence AF222068.[21]

The *A. fumigatus cyp51A* sequence AF338659[22] was obtained from the universal protein resource (http://www.uniprot.org). Homology modelling was *inter alia cyp51B*-based with the crystal structure of *A. fumigatus cyp51B* retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org) as it returned the highest sequence identity and BLAST score (identification no. 5FRB: resolution 2.99 Å, max score 660, total score 660, query cover 92%, identity 65%). Of five homology models of the *cyp51A* gene built using Schrödinger's Prime,[23] the best model (data not shown) was prepared for molecular docking (see below).

The single-residue mutation G54R was tested with 2D structures of itraconazole, posaconazole and voriconazole and drawn (Schrödinger Release 2017-3; LigPrep LLC, New York). Docking of the three triazoles was carried out via Glide with extra precision (XP),[24] and the potential energy in different conformations with an open and closed binding cavity was calculated via MacroModel (Schrödinger Release 2017-3).

Results

Environmental sampling

In addition to culture collection isolates included in this study [ambient air ($n = 6$) and soil ($n = 20$)] a further 159 *A. fumigatus* environmental cultures were isolated from soil. Method 1 yielded 91 *A. fumigatus* isolates from azole-naive soils ($n = 90$) and azole-exposed soils ($n = 1$). Method 2 yielded an additional 68 isolates from azole-naive soils ($n = 5$) and azole-exposed soils ($n = 63$).

Resistance screening

Five isolates (one avian and four from azole-exposed soil) grew on the itraconazole-containing well of the VIPcheck™ plate. Of 148 human isolates, isolate 129 grew on all azole-supplemented wells, and isolates 9 and 32 grew on posaconazole- and itraconazole-containing wells. Isolates 32 and 129 were from patients who had received prior fluconazole as antifungal prophylaxis during the preceding 6 month period, whilst isolate 9 was from an azole-naive patient. All three patients had invasive pulmonary aspergillosis.[12]

Susceptibility testing

Table 1 summarizes the MIC/MEC values for the study isolates (see Tables S1 and S2 for individual isolate results). Of 148 human isolates, 145 had WT MICs below the ECV for the drugs tested with GM MICs of itraconazole, posaconazole, voriconazole and amphotericin B of 0.12, 0.06, 0.23 and 1.04 mg/L, respectively (MIC_{90} values were 0.25, 0.12, 0.5 and 2.0 mg/L, respectively). Of three isolates that had MICs at the ECV or NWT MICs (Table S1), isolates 9 and 32 had an MIC of 1 mg/L of posaconazole and itraconazole (voriconazole MIC 0.12 mg/L). Isolate 129 had MICs of >16, 8 and 1 mg/L of itraconazole, voriconazole and posaconazole,

Table 1. Antifungal susceptibilities [MIC or MEC[a] values (mg/L)] of 147 human,[b] 11 animal and 41 environmental isolates of *A. fumigatus*, according to origin of isolates

	AMB	ANF	CAS	FLC	ITC	MIF	POS	VRC
GM MIC/MEC								
human, invasive (35)	1.2	0.02	0.04	184.35	0.22	0.01	0.11	0.28
human, colonization (112)	0.99	0.02	0.03	195.85	0.12	0.01	0.05	0.22
animal (11)	1.13	0.02	0.04	256	0.12	0.02	0.04	0.34
azole-exposed soil (23)[c]	1.2	0.02	0.04	246	0.13	0.01	0.06	0.27
azole-naive soil (12)	1.06	0.02	0.04	256	0.12	0.02	0.04	0.31
air (6)	2	0.015	0.04	228	0.15	0.01	0.05	0.25
MIC/MEC_{90}								
human, invasive (35)	2	0.03	0.12	>256	0.5	0.015	0.5	0.5
human, colonization (112)	2	0.03	0.06	>256	0.25	0.03	0.12	0.5
animal (11)	2	0.03	0.06	>256	0.25	0.03	0.12	0.5
azole exposed soil (23)[c]	2	0.03	0.06	>256	0.25	0.015	0.12	0.5
azole-naive soil (12)	1	0.03	0.06	>256	0.25	0.03	0.12	0.5
air (6)	2	0.015	0.06	256	0.25	0.015	0.06	0.5
MIC/MEC range								
human, invasive (35)	0.25–8	<0.015–0.06	0.008–0.5	64 to >256	0.06 to >16	<0.008–0.03	0.015–1	0.06–8
human, colonization (112)	0.25–2	<0.015–0.12	<0.008–0.12	64 to >256	<0.015–0.5	<0.008–0.03	<0.008–0.25	0.03–1
animal (11)	0.5–2	0.015–0.03	0.015–0.06	256 to >256	0.03–0.5	0.008–0.03	0.015–0.25	0.25–0.5
azole-exposed soil (23)[c]	0.5–2	0.015–0.06	0.015–0.06	128 to >256	0.03–0.5	0.008–0.03	0.015–0.25	0.12–1.0
azole-naive soil (12)	1–2	0.015–0.06	0.03–0.06	256 to >256	0.03–0.25	0.008–0.03	0.015–0.12	0.25–0.5
air (6)	2	0.015	0.03–0.06	128–256	0.12–0.25	0.008–0.015	0.03–0.06	0.06–0.5

AMB, amphotericin B; ANF, anidulafungin; CAS, caspofungin; FLC, fluconazole; ITC, itraconazole; MIF, micafungin; POS, posaconazole; VRC, voriconazole.
Numbers in brackets refer to the numbers of isolates tested.
[a]MECs are reported for echinocandin drugs (anidulafungin, caspofungin and micafungin).
[b]Disease state information was not available for one human isolate (isolate number 24). MICs for this isolate were (in mg/L): amphotericin B, 1; anidulafungin, 0.015; caspofungin, 0.06; fluconazole, 256; itraconazole, 0.25; micafungin, 0.008; posaconazole, 0.12; and voriconazole, 0.25.
[c]DMIs used on azole-exposed soil isolates included cyproconazole ($n = 3$), myclobutanil ($n = 4$), tebuconazole and epoxiconazole ($n = 10$), tebuconazole and myclobutanil ($n = 1$), tebuconazole and penconazole ($n = 3$), and tetraconazole and myclobutanil ($n = 2$).

respectively. However, the avian isolate that grew on the itraconazole-containing well of the VIPcheck™ plate had a WT itraconazole MIC (0.06 mg/L). The assignment of MIC results as WT or NWT/MIC at the ECV of clinical isolates was concordant with results obtained by VIPcheck™ screening for 99% of clinical isolates.

All 41 environmental isolates had WT MIC values of the azoles (Table 1); GM MICs of itraconazole, posaconazole and voriconazole were 0.13, 0.05 and 0.29 mg/L, respectively (MIC_{90} 0.25, 0.12 and 0.5 mg/L). All four isolates that grew on the itraconazole-containing agar well of the VIPcheck™ plate had WT MICs of itraconazole [0.06 ($n = 3$) and 0.12 ($n = 1$) mg/L]. Comparison of GM MICs, MIC_{50} values and MIC_{90} values between 'azole-exposed' and 'azole-naive' isolates showed these were not statistically different (data not shown: posaconazole, $P > 0.05$–0.1; and itraconazole and voriconazole, $P > 0.2$).

cyp51A sequencing and modelling

cyp51A mutations were identified in the three isolates with NWT MICs or MICs at the ECV (see Table S1) (GenBank accession numbers MG687254–MG687257). Isolates 9 and 32 had SNPs present at nucleotide position 160 (GGG to AGG and CGG, respectively), resulting in a G54R substitution. Isolate 32 had another SNP at nucleotide position 744 (AAT to AAA), resulting in N248K. Isolate 129 carried a TR_{34}/L98H mutation at nucleotide position 323.

Modelling G54R indicated that replacing the short side-chain glycine (G) with arginine (R) allowed the formation of hydrogen bonds with T50 and S493, which closes off binding channel access (Figure 1). This closed conformation stabilized the model by 84.47 kcal/mol with high likelihood that the large triazole structures (itraconazole and posaconazole but not voriconazole) would be prevented from entering the channel.

Discussion

This large study found isolates with NWT MICs to be uncommon in Australian human clinical isolates (2.0%), and at the lower end of that seen globally (0%–26%), contrasting particularly with that in the Netherlands.[5,8,10] Of note, ARAf was absent amongst animal isolates. These data suggest that treatment guidelines currently recommending azoles as preferred therapy for IA remain appropriate in Australia.[3]

Also notable was the apparent absence of ARAf in the environment, including azole-exposed habitats. Environmental studies

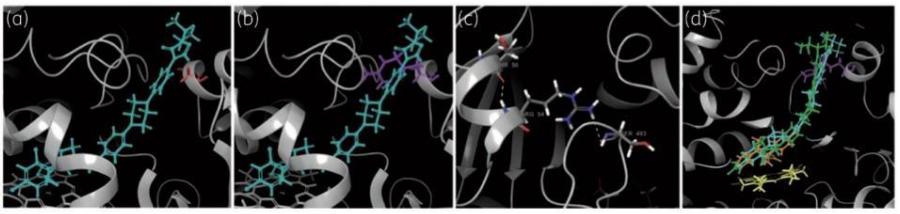


Figure 1. *A. fumigatus cyp51B* model demonstrating binding to the active site of *cyp51A* and the H-bond interaction. Itraconazole (blue) binding in the active site of *cyp51A*: (a) amino acid G54 (red) and (b) mutated G54R (purple). (c) Hydrogen bonding (yellow dashed lines) between T50, G54R and S493 (H-bond length: T50 and G54R, 1.99 Å; and S493 and G54R, 2.28 Å). (d) Voriconazole (orange), posaconazole (green) and itraconazole (blue) in the binding cavity; residue G54R (purple) and the haem group (yellow) are shown.

have reported a broad range of prevalence of ARAf (0%–91%), with the highest prevalence in the Netherlands, where most resistant isolates carry the TR_{34}/L98H mechanism.[7] The implications of our results for agricultural practice, although uncertain, raise questions regarding the impact of different agricultural practices between Australia and Europe including percentage of crop area treated, number and amount of applications/crop, and type and rotation of DMIs used. Composts, which have yielded a high percentage of environmental ARAf in Europe,[25] are not used in broad-acre farming in Australia. Additionally, compared with Western Europe, the Australian climate has prolonged periods of dryness or drought, where the need for DMIs is greatly reduced. In Australia, the most commonly used azole fungicides for grape crops include difenoconazole, myclobutanil, penconazole, tebuconazole and tetraconazole and for grain crops cyproconazole, epoxiconazole, propiconazole, prothioconazole and tebuconazole.[26] Azole fungicide and medical practices of different locations worldwide should be compared to further our understanding of the variation in ARAf prevalence rates between regions.

The agar-based VIPcheck™ screening test is well established to have sufficiently high sensitivity and specificity for predicting azole resistance.[27] In the present study, the test proved useful in screening for resistance with no false 'susceptible' results; all isolates with NWT MICs or with MICs at the ECV were observed to grow on the plates. The VIPcheck™ plates overcalled 'resistance' for five isolates (5/8 isolates, specificity 97.5%). This is reasonable for a screening test which must have a high negative predictive value. As the VIPcheck™ test does not measure MICs, all isolates that grow on azole-supplemented agar should be tested for antifungal susceptibility.

The G54R mutation, identified here in two isolates, is known to confer differential azole resistance in *A. fumigatus*.[9] One of our isolates with the G54R mutation had a second mutation leading to amino acid substitution N248K; however, this also occurs in azole-susceptible *A. fumigatus* isolates with no documented link to resistance.[9] Pan-resistance in isolate 129 was through the TR_{34}/L98H mutation, consistent with existing literature.[10] Of note, both TR_{34}/L98H and G54R mutations have been linked to environmental acquisition,[7,10] and were identified previously in three other Australian clinical isolates.[11]

Our homology modelling results using a *cyp51B A. fumigatus* model are consistent with previous observations in non-*Aspergillus cyp51A* models that the G54R mutation can prevent docking of the long-tailed azoles itraconazole and posaconazole but not of the more compact voriconazole molecule.[28]

Recovery of *A. fumigatus* from soil found that method 2, which uses heat-shock to inactivate asexual spores, gave a higher yield of isolates from azole-treated soils. Although no ARAf was found, the survival of sexual spores in azole-treated soil could serve as a potential reservoir for resistance development; in the laboratory setting, sexual reproduction has been implicated in the development of *cyp51A*-mediated azole resistance.[25]

A limitation of the present study was that clinical data were not collected. This prevented more precise determination of ARAf incidence amongst different groups of azole-exposed or azole-naive patients. About half of the cystic fibrosis patients in the Centre for Infectious Diseases and Microbiology Laboratory Services receive intermittent azole therapy (usually itraconazole) (S. C.-A. Chen, unpublished data). Other isolates were recovered from disparate groups of patients with unknown azole exposure. Whilst it is possible that the low prevalence observed is related to potential inhomogeneity in the patient population, the fact that we only observed three 'azole-resistant' isolates would limit any statistically meaningful data. We acknowledge the importance of prospective studies to investigate azole resistance amongst specific patient groups.

In conclusion, ARAf appears to be uncommon in Australia, which indicates that the preferred therapy for aspergillosis does not need to be changed. However, continued surveillance is warranted to pre-empt the emergence of ARAf strains and to inform management strategies.

Acknowledgements

We wish to thank Professor Paul Verweij and the Department of Medical Microbiology at the Institute for Molecular Life Sciences at Radboud UMC, Nijmegen, The Netherlands, and Professor Oliver Bader at the Institute of Medical Microbiology, University Medical Center Goettingen, Göttingen, Germany, for providing assistance with aligning nucleotide positions to pinpoint mutation locations.

Funding

The study was supported in part by an untied educational grant to S. C.-A. C. and V. R. B. from MSD Australia, and by an intramural grant to V. R. B. from the Sydney School of Veterinary Science. T. C. S. is a Sydney Medical Foundation Fellow.

Transparency declarations

C. L. H.: untied educational grants from MSD Australia. T. C. S.: untied educational grants from MSD Australia; and Antifungal Advisory Board for Pfizer Australia, Gilead Sciences Inc. and Astellas. V. R. B.: untied educational grants from MSD Australia. S. C.-A. C.: untied educational grants from MSD Australia; Antifungal Advisory Board for Pfizer Australia, Gilead Sciences Inc. and Astellas; and Speakers' Bureau for MSD Australia. All other authors: none to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 Brown GD, Denning DW, Gow NA *et al*. Hidden killers: human fungal infections. *Sci Transl Med* 2012; **4**: 165rv13.

2 Steinmann J, Hamprecht A, Vehreschild MJ *et al*. Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. *J Antimicrob Chemother* 2015; **70**: 1522–6.

3 Patterson TF, Thompson IIIGR, Denning DW *et al*. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis* 2016; **63**: e1–60.

4 Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. The global problem of antifungal resistance: prevalence, mechanisms, and management. *Lancet Infect Dis* 2017; **17**: e383–92.

5 van der Linden JW, Arendrup MC, Warris A *et al*. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg Infect Dis* 2015; **21**: 1041–4.

6 van der Linden JW, Snelders E, Kampinga GA *et al*. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. *Emerg Infect Dis* 2011; **17**: 1846–54.

7 Verweij PE, Snelders E, Kema GHJ *et al*. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect Dis* 2009; **9**: 789–95.

8 Garcia-Rubio R, Cuenca-Estrella M, Mellado E. Triazole resistance in *Aspergillus* species: an emerging problem. *Drugs* 2017; **77**: 599–613.

9 Dudakova A, Spiess B, Tangwattanachuleeporn M *et al*. Molecular tools for the detection and deduction of azole antifungal drug resistance phenotypes in *Aspergillus* species. *Clin Microbiol Rev* 2017; **30**: 1065–91.

10 Chowdhary A, Sharma C, Meis JF. Azole-resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. *J Infect Dis* 2017; **216** Suppl 3: S436–44.

11 Kidd SE, Goeman E, Meis JF *et al*. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses* 2015; **58**: 350–5.

12 De Pauw B, Walsh TJ, Donnelly JP *et al*. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis* 2008; **46**: 1813–21.

13 Snelders E, Huis in 't Veld RA, Rijs AJM *et al*. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazole. *Appl Environ Microbiol* 2009; **75**: 4053–7.

14 Nováková A, Hubka V, Dudová Z *et al*. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. *Fungal Divers* 2014; **64**: 253–74.

15 Samson RA, Houbraken J, Thrane U *et al*. *Food and Indoor Fungi*. Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures, 2010.

16 Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* 1995; **61**: 1323–30.

17 van der Linden JWM, Arendrup MC, van der Lee HAL *et al*. Azole containing agar plates as a screening tool for azole resistance of *Aspergillus fumigatus*. *Mycoses* 2009; **52**: 19.

18 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi—Third Edition: M38*. CLSI, Wayne, PA, USA, 2017.

19 Espinell-Ingroff A, Diekema DJ, Fothergill A *et al*. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *J Clin Microbiol* 2010; **48**: 3251–7.

20 Clinical and Laboratory Standards Institute. *Epidemiology Cutoff Values for Antifungal Susceptibility Testing—Second Edition: M59*. CLSI, Wayne, PA, USA, 2018.

21 Chen J, Li H, Li R *et al*. Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *J Antimicrob Chemother* 2005; **55**: 31–7.

22 Mellado E, Diaz-Guerra TM, Cuenca-Estrella M *et al*. Identification of two different 14-α sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *J Clin Microbiol* 2001; **39**: 2431–8.

23 Jacobson MP, Pincus DL, Rapp CS *et al*. A hierarchical approach to all-atom protein loop prediction. *Proteins* 2004; **55**: 351–67.

24 Friesner RA, Murphy RB, Repasky MP *et al*. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J Med Chem* 2006; **49**: 6177–96.

25 Zhang J, Snelders E, Zwaan BJ *et al*. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *MBio* 2017; **8**: e00791-17.

26 Australian Wine Research Institute. *Agrochemicals Registered for Use in Australian Viticulture*. 2017. https://www.awri.com.au/wp-content/uploads/agrochemical_booklet.pdf.

27 Arendrup MC, Verweij PE, Mouton JW *et al*. Multicentre validation of 4-well azole agar plates as a screening method for detection of clinically relevant azole-resistant *Aspergillus fumigatus*. *J Antimicrob Chemother* 2017; **72**: 3325–33.

28 Fraczek MG, Bromley M, Bowyer P. An improved model of the *Aspergillus fumigatus* CYP51A protein. *Antimicrob Agents Chemother* 2011; **55**: 2483–6.

Appendix 6: Disseminated *Scedosporium prolificans* infection in a Labrador retriever with immune mediated haemolytic anaemia

6.1 Introduction

The following appendix contains a publication by Taylor et al., 2014 (http://dx.doi.org/10.1016/j.mmcr.2014.10.001). This publication details a clinical case of disseminated scedosporiosis in a dog (Labrador retriever) that presented to the University Veterinary Teaching Hospital Sydney for diagnosis and treatment. The case was managed by Dr A. Taylor under the supervision of Prof. V.R. Barrs and Dr P. Bennett.

I performed the molecular identification on the clinical samples, identifying the agent of disease as *Scedosporium prolificans*, and assisted in drafting and finalising the manuscript.

6.2 Main article

Medical Mycology Case Reports 6 (2014) 66–69

Contents lists available at ScienceDirect

Medical Mycology Case Reports

journal homepage: www.elsevier.com/locate/mmcr

Disseminated *Scedosporium prolificans* infection in a Labrador retriever with immune mediated haemolytic anaemia

Amanda Taylor, Jessica Talbot, Peter Bennett, Patricia Martin, Mariano Makara, Vanessa R. Barrs *

Faculty of Veterinary Science, University of Sydney, Sydney NSW 2006, Australia

ARTICLE INFO

Article history:
Received 28 August 2014
Received in revised form
28 September 2014
Accepted 27 October 2014

Keywords:
Canine
Scedosporiosis
Invasive fungal infection

ABSTRACT

Disseminated scedosporiosis is rare in dogs and is usually reported in German Shepherds with suspected heritable immunodeficiency. This is the first report of disseminated scedosporiosis due to *Scedosporium prolificans* in a Labrador retriever dog that was receiving immunosuppressive drug therapy for treatment of immune-mediated haemolytic anaemia. Despite cessation of immunosuppressive medications and an initial response to aggressive treatment with voriconazole and terbinafine the dog developed progressive disease with neurological signs necessitating euthanasia six months from diagnosis.

1. Introduction

Infections caused by fungi belonging to the *Scedosporium*/*Pseudallescheria* complex (SPCF) in dogs are restricted to a small number of cases including localised infections involving the skin, upper respiratory tract and eyes and disseminated disease [1]. *Scedosporium prolificans* and *Scedosporium apiospermum* (teleomorph *Pseudallescheria boydii*) are the medically significant species in the SPCF [2]. We report the first case of disseminated mycosis due to *S. prolificans* in a Labrador retriever dog that was receiving immunosuppressive drug therapy for treatment of immune mediated haemolytic anaemia (IMHA).

2. Case

A four-year-old male desexed Labrador retriever was referred to the University Veterinary Teaching Hospital, Sydney (UVTHS) with a four week history of right hind limb lameness and a one week history of multiple ulcerated skin lesions. Ten months previously the dog had been diagnosed with primary immune mediated haemolytic anaemia (IMHA) and was treated with immunosuppressive medication (prednisolone 1.5 mg/kg PO every 12 h; azathioprine 1.25 mg/kg PO every 24 h), and antiplatelet therapy (aspirin 0.6 mg/kg PO every 24 h). Following tapering of immunosuppressive medication, the dog represented to the UVTHS three months after initial diagnosis (day −176) with severe recurrent IMHA. Prednisolone (1 mg/kg PO every 12 h) and azathioprine (1.25 mg/kg PO every 24 h) were again prescribed with the addition of cyclosporine (5.5 mg/kg PO every 24 h). The dog responded well and all drug dosages were tapered slowly over the following seven months (prednisolone 0.125 mg/kg PO every second day; azathioprine 1.25 mg/kg PO every other day). Cyclosporine was discontinued three months (day −97) prior to presentation.

On presentation to the UVTHS on day 0 the dog weighed 39.7 kg, was in good body condition and had a normal heart rate, respiratory rate and rectal temperature. It was lame (grade III/V) on the right hind limb and had multiple ulcerated skin lesions with draining sinus tracts over the head, neck and body (Fig. 1). Pain was elicited on manipulation of the right coxo-femoral joint and mid thoracic spine. Pitting oedema was present distal to the right hock. Mild bilateral elbow effusion was detected. The left pre-scapular and popliteal lymph nodes were palpably enlarged.

Given the clinical presentation and previous history of IMHA, differential diagnoses included disseminated bacterial or fungal infection, immune mediated polyarthropathy and dermatopathy as an indication of multisystemic autoimmunity, or systemic lupus erythematous.

A complete blood count (CBC) and serum biochemistry were performed. There was a mild non-regenerative anaemia (haematocrit 0.32 L/L; reference interval: 0.37–0.55 L/L), moderate leucocytosis (23.4×10^9 /L; reference interval: $7–12 \times 10^9$ /L) primarily due to a neutrophilia (18.95×10^9 /L; reference interval: $4.06–9.36 \times 10^9$ /L) with a left shit (bands 0.23×10^9 /L; reference interval: $0–0.24 \times 10^9$ /L) and a mild monocytosis (1.64×10^9 /L; reference interval: $0.21–0.96 \times 10^9$ /L). Mild elevations in alkaline phosphatase

* Corresponding author. Tel.: +61 2 9351 3437; fax: +61 2 9351 7436.
E-mail address: vanessa.barrs@sydney.edu.au (V.R. Barrs).

http://dx.doi.org/10.1016/j.mmcr.2014.10.001

(ALP 261 U/L; reference interval: < 110 U/L), alanine transaminase (ALT 86 U/L; reference interval: < 60 U/L), total protein (72.8 g/L; reference interval: 50–70 g/L) and phosphate (2.02 mmol/L; reference interval: 0.8–1.6 mmol/L) were noted on serum biochemistry. In addition, total serum calcium (3.43 mmol/L; reference interval: 2.1–2.9 mmol/L) was elevated and ionised calcium was normal (1.28 mmol/L; reference interval: 1.25–1.5 mmol/L). An antinuclear antibody (ANA) titre was negative (< 1:25).

Abdominal ultrasound demonstrated increased, but homogeneous echogenicity of the pancreas, consistent with chronic pancreatitis and mild bilateral renal pelvis dilation. On urinalysis short septate branching fungal hyphae were detected. Whole body computed tomography (CT) on day 2 revealed multiple productive bone lesions in the distal portion of the third and eight ribs; lysis of the opposing endplates at thoracic vertebrae 5–6 and sternebrae 2–3 with surrounding bone sclerosis; and irregular periosteal new bone formation associated with the metatarsal and tarsal bones of the right hind limb. The right liver lobes had irregular borders and diffusely heterogeneous post-contrast enhancement in the arterial phase. In addition, generalised abdominal, sternal and peripheral lymphadenopathy was seen (Fig. 2). Cytological examination of joint fluid from the right hock and carpus was unremarkable. Fine-needle aspirate biopsies from rib and sternebrae lesions, and impression smears of multiple skin lesion biopsies showed marked pyogranulomatous inflammation. Fungal hyphae were identified in skin lesions.

Itraconazole (5 mg/kg PO twice daily) was prescribed on day 3 whilst awaiting fungal culture results and the dog was discharged from hospital. Immunosuppressive medication doses were reduced then stopped after 5 days.

A suede-like black pigmented fungus was cultured on Sabouraud's dextrose agar on day 7 at 28 °C from urine, bone and skin, but not from joint fluid. On microscopic examination, septate hyphae had basally inflated flask shaped conidiophores with small clusters of conidia consistent with *S. prolificans*. A PCR targeting the partial β-tubulin gene was performed using DNA extracted from fungal culture material, as previously described [3]. The molecular identity was *S. prolificans* (99% homology with GenBank accession number AJ889591.1) [4]. The isolate was submitted to the Australian Reference Laboratory in Medical Mycology, Adelaide for susceptibility testing (Table 1).

Histopathology of skin revealed multifocal pyogranulomatous dermal inflammation with brown pigmented periodic acid-Schiff (PAS) positive irregularly septate hyphae occasionally branching at 90° within inflammatory foci.

Over a three week period the skin lesions improved. Terbinafine (30 mg/kg PO once daily) was added to the treatment regimen on day 16. Following antifungal susceptibility testing results, voriconazole (5 mg/kg PO twice daily), obtained from a veterinary compounding pharmacy was substituted for itraconazole (day 35). The dog was continued on voriconazole and terbinafine with no clinical side effects noted. After 6 weeks of treatment, all skin lesions had resolved and the hind limb lameness had improved. The dog's haematocrit had increased from 0.31 to 0.37 L/L indicating that the IMHA was in remission. Repeat serum biochemistry was unremarkable.

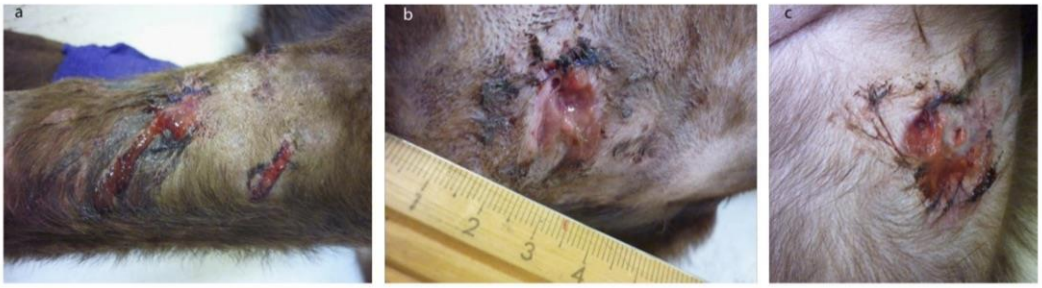


Fig. 1. Ulcerated skin lesions with draining sinus tracts. (a) Left antebrachium. (b) Right lateral aspect of the neck. Similar lesions were also seen on the left side of the neck, at the base of the right ear and at the lip commissures; and (c) medial aspect of right hind limb.

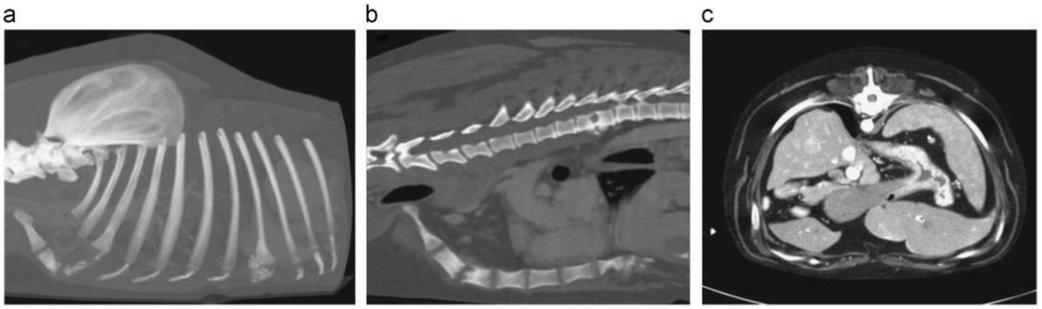


Fig. 2. CT images. (a) Sagittal volume rendering maximum intensity projection reconstruction of the thorax. On the right side of the thorax, a productive lesion, associated with irregular new bone formation can be seen affecting the distal aspect of the third and eight rib at the level of the costochondral junction. (b) Sagittal reconstructed image of the thorax. Lysis of the opposing end-plates of thoracic vertebrae 5–6 and sternebrae 2–3. There is increased bone opacity surrounding the lytic end-plates. Spondylosis deformans can be seen associated with the T5–6 lesion. (c) Axial image of the abdomen at the level of the liver acquired during the arterial phase. Right side of the liver presents heterogeneous enhancement and irregular contours. A 15 mm hypoattenuating lesion can be seen in the caudate lobe during the arterial phase only.

Table 1
Antifungal susceptibility testing results.

Antifungal	MIC (mg/L)	R/S/I/SDD[a]
Amphotericin B	4.0	R
5-Fluorocytosine	> 64	R
Fluconazole	> 256	R
Itraconazole	> 16	R
Voriconazole	4.0	R
Posaconazole	> 8.0	R
Caspofungin	> 8.0	R
Anidulafungin	> 8.0	R
Micafungin	> 8.0	R

R = resistant.
I = intermediate.
SDD = susceptible dose dependent.
[a] S = susceptible.

The dog was readmitted to hospital approximately 3 months (day 101) later for a repeat CT evaluation, CBC, serum biochemistry and urine culture. At this time, despite resolution of the right hind limb lameness, the dog had lost 6.7 kg since diagnosis. The haematocrit (0.49 L/L) and leucocyte count (9.0×10^9 /L) were normal. There was moderate hypoalbuminaemia (19.7 g/L; reference interval: 23–43 g/L) with normal total protein (61.5 g/L; reference interval: 50–70 g/L) and globulins (41.8 g/L; reference interval: 27–44 g/L). On repeat CT, bone, liver and lymph node lesions were unchanged. Repeat urine culture was positive for *S. prolificans*.

A urine protein creatinine ratio was normal (0.2; reference interval: 0.2–0.5), as were pre- and post-prandial bile acids. Hypoalbuminaemia was attributed to gastrointestinal losses due to mycotic involvement or concurrent inflammatory bowel disease. The owner was reluctant to pursue further diagnostic investigations. Antifungal drugs were continued and the dog was transitioned to a hypoallergenic diet.

The dog remained clinically well for another seven weeks before representing three times over a five week period for recurrent inappetence, pyrexia, dehydration and altered behaviour. On each occasion, the dog was treated conservatively with intravenous fluids and discharged within 24 h. Repeat biochemistry indicated worsening hypoalbuminaemia (13.8 g/L). During this time the owner sought a second opinion at another small animal specialist practice. A rectal scape demonstrated the presence of fungal hyphae, further mycotic involvement of the gastrointestinal tract.

Six months (day 183) following diagnosis, the dog represented with inappetence and lethargy. Twelve hours following admission, the dog's neurological status rapidly deteriorated with abnormalities suggestive of a C1–T2 spinal cord segment or brain stem lesion. It became obtunded, hyperthermic, tachycardic and developed an erratic breathing pattern. Given the rapid deterioration and poor prognosis, the owners elected to humanly euthanase the dog. A full post mortem was not conducted. Cerebrospinal fluid collected immediately following euthanasia indicated a mild mononuclear pleocytosis. Fungal culture was negative.

3. Discussion

This is the first report of disseminated mycosis due to *S. prolificans* in a Labrador retriever dog. Previous reports of disseminated scedosporiosis in dogs have been restricted to German Shepherd dogs (GSD) [5–9], and were due to *S. apiospermum* infection [5,6,8] except in one case of *S. prolificans* infection [7]. There is a single case reported of disseminated *S. prolificans* (previously *S. inflatum*) infection in another breed; a Beagle with osteomyelitis and pulmonary involvement [9].

Scedosporiosis is considered an emerging disease in human medicine. In contrast to *S. apiospermum*, which is commonly found in soil, sewage and polluted waterways, *S. prolificans* is more commonly isolated from soil and potted plants and has a more restricted geographical distribution with the majority of cases in humans reported in Australia and Spain [2]. Interestingly, one of the two previously reported cases of disseminated *S. prolificans* infection in dogs also occurred in Australia [7], with the other occurring in New York [9]. Scedosporiosis in humans represents a broad spectrum of clinical disease including local cutaneous and subcutaneous infection (eumycetoma) that occur secondary to penetration wounds; saprobic involvement of a previously damaged bronchopulmonary tree; invasive pulmonary disease; sinopulmonary and central nervous system infection associated with near drowning's in polluted water ways contaminated with *P. boydii*; and disseminated infection. Except for eumycetomas and near drowning's infection occurs in people with immune suppression or immune dysfunction. More specifically, disseminated infections are most commonly reported in humans with disorders in innate immunity such as chronic granulomatous disease or in those receiving immunosuppressive drug therapy as a result of haematopoietic malignancies and bone marrow or solid organ transplants [2].

GSD have an increased susceptibility to disseminated fungal infections, including aspergillosis and scedosporiosis. Abnormalities in IgA regulation and function, and dysfunction of cell mediated immunity is suspected [10]. No such immune deficiency has been identified in Labrador retrievers. In the Beagle with disseminated scedosporisosis, no underlying immune deficiency or history of immune suppressive medication was noted. In our case, it is likely that immune suppression induced by treatment for IMHA allowed for opportunistic infection with *S. prolificans*. The use of glucocorticoids is a well-documented risk factor for developing invasive fungal infections [2]. Glucocorticoids therapy results in global immune suppression and the risk of infection increases with increasing dose and duration of treatment beyond 21 days [11]. A history of corticosteroid use and persistent profound neutropenia have been identified as risk factors in developing disseminated *S. prolificans* infection in humans [2]. Cyclosporine also has potent immunosuppressive activity, acting by interrupting intracellular signalling, resulting in significant negative effects on T-cell function and cell medicated immunity. Invasive fungal infections are a common complication in humans treated with cyclosporine following solid organ transplant [12]. In our case, the dose of prednisolone was low at the time of presentation; however the prolonged duration of therapy, initial high doses and combination therapy with azathioprine and cyclosporine, likely contributed to significant immunosuppression. A similar recent case report from Australia describes a 2 year male desexed Rhodesian Ridgeback dog that developed lymphocutaneous lesions due to *S. apiospermum* infection whist receiving cyclosporine, azathioprine and prednisolone for IMHA and immune mediated polyarthritis [13]. In contrast to our case, once immunosuppressive medication was discontinued, the dog was able to clear the infection.

The route of infection in our case is not clear. Humans with disseminated *S. prolificans* have a high rate of fungaemia that results in fungal embolization of multiple organs. Haematogenous spread from primary pulmonary infection is the most common cause of fungaemia, although cutaneous lesions have been noted to precede dissemination in some cases [2]. No clear route of infection has been identified in previously reported cases of disseminated disease in dogs. In several cases with osteomyelitis [5,6,9], a penetrating wound or foreign body was suspected but unproven. In our case, the dog had ulcerated skin lesion noted five months prior to presentation. The lesions were noted on the forelimbs associated with areas of pressure or wear and were considered likely due to the effects of prednisolone therapy on the skin namely thinning and increased fragility. The skin lesions had

healed six weeks prior to initial presentation however this break in integument may have been the route of entry for the fungal infection. The dog had no clinical sings attributable to pulmonary disease and mild changes noted on the CT scan of the thorax where more consistent with atelectasis rather than pneumonia. It seems less likely that dissemination occurred from primary pulmonary disease however this cannot be excluded as previous case reports have only noted disease involving the lungs at the time of post mortem examination [7,9]. Polyphagia and pica are common side effects seen in dogs receiving prednisolone. Given this, entry through the gastrointestinal tract as a result of indiscriminate eating patterns is also possible in this case. Gastrointestinal fungal involvement was demonstrated on rectal scape that would support this.

There is little available information regarding successful treatment of disseminated scedosporiosis in dogs. Treatment with itraconazole was attempted in three previous cases with no success; two dogs were euthanaised within a few weeks of treatment due to lack of improvement [6,9] and the third dog died acutely 5 days after initiation of treatment [7]. The case of lymphocutaneous infection due to *S. apiospermum* was successfully treated with itraconazole and terbinafine and cessation of immunosuppressive medication. Success in this case may be due to several reasons; removal of the cause of immunosuppression in an otherwise immunocompetent individual; restriction of infection to the skin; and a less virulent, more anti- fungal responsive isolate, *S. apiospermum*.

Mortality rates as high as 90% are reported in human with disseminated *S. prolificans* infection [14]. *S. prolificans* has a high degree of antifungal resistance with high minimum inhibitory (MICs) values to the majority of available antifungal agents. However, combination therapy using terbinafine with voriconazole, itraconazole or miconazole has been shown to result in synergy in vitro against human clinical isolates of *S. prolificans* such that MIC values were reduced to clinically achievable levels [15]. In vitro synergy has also been reported with combinations of amphotericin B and micafungin [16], and voriconazole and micafungin [17]. Successful treatment has been reported in vivo in clinical cases using combination antifungal therapy with terbinafine and voriconazole [18–20]. However aggressive surgical debridement and/or immune modulation therapy was often used to complement antifungal therapy. Combination antifungal susceptibility testing is not easily available in Australia. Thus information was extrapolated from studies in humans and used to guide the choice of combination antifungal treatment prescribed in our dog. Despite high in vitro MIC (> 16 mg/L) for itraconazole, significant and sustained clinical improvement occurred for six months after treatment with combination therapy using itraconazole then voriconazole, combined with terbinafine. It is also possible that voriconazole, sourced from a compounding pharmacy, was not as efficacious as the non-generic formulation of the drug.

In conclusion, we present a novel case of a Labrador retriever that developed disseminated *S. prolificans* infection secondary to immunosuppressive drug therapy and demonstrate a partial clinical response to combination therapy with voriconazole and terbinafine.

Conflict of interest statement

None.

References

[1] Elad D. Infections caused by fungi of the *Scedosporium*/*Pseudallescheria* complex in veterinary species. Vet J 2011;187(1):33–41.
[2] Cortez KJ, Roilides E, Quiroz-Telles F, Meletiadis J, Antachopoulos C, Knudsen T, et al. Infections caused by Scedosporium spp. Clin Microbiol Rev 2008;21(1):157–97.
[3] Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 1995;61(4):1323–30 (April 1).
[4] Gilgado F, Cano J, Gené J, Guarro J. Molecular phylogeny of the *Pseudallescheria boydii* species complex: proposal of two new species. J Clin Microbiol 2005;43(10):4930–42.
[5] Baszler T, Chandler F, Bertoy R, Smith C, Whiteley H. Disseminated pseudallescheriasis in a dog. Vet Pathol Online 1988;25(1):95–7.
[6] Elad D, Perl S, Yamin G, Blum S, David D. Disseminated pseudallescheriosis in a dog. Med Mycol 2010;48(4):635–8.
[7] Haynes S, Hodge P, Tyrrell D, Abraham L. Disseminated *Scedosporium prolificans* infection in a German Shepherd dog. Aust Vet J 2012;90(1–2):34–8.
[8] Hugnet C, Marrou B, Dally C, Guillot J. Osteomyelitis and discospondylitis due to *Scedosporium apiospermum* in a dog. J Vet Diagn Investig 2009;21(1):120–3.
[9] Salkin I, Cooper C, Bartges J, Kemna M, Rinaldi M. Scedosporium inflatum osteomyelitis in a dog. J Clin Microbiol 1992;30(11):2797–800.
[10] Day MJ, Penhale WJ. An immunohistochemical study of canine disseminated aspergillosis. Aust Vet J 1991;68(12):383–6.
[11] Cutolo M, Seriolo B, Pizzorni C, Secchi ME, Soldano S, Paolino S, et al. Use of glucocorticoids and risk of infections. Autoimmun Rev 2008;8(2):153–5.
[12] Hadley S, Samore MH, Lewis WD, Jenkins RL, Karchmer AW, Hammer SM. Major infectious complications after orthotopic liver transplantation and comparison of outcomes in patients receiving cyclosporine or FK506 as primary immunosuppression. Transplantation 1995;59(6):851–8.
[13] Brown J, Bennett P, Labuc R, Merrett D. Lymphocutaneous infection with *Scedosporium apiospermum* in a dog on immuno-suppressant therapy. Aust Vet Pract 2009;39(2):50–4.
[14] Revankar SG, Patterson JE, Sutton DA, Pullen R, Rinaldi MG. Disseminated phaeohyphomycosis: review of an emerging mycosis. Clin Infect Dis 2002;34(4):467–76 (February 15).
[15] Meletiadis J, Mouton JW, Meis JF, Verweij PE. In vitro drug interaction modeling of combinations of azoles with terbinafine against clinical *Scedosporium prolificans* isolates. Antimicrob Agents Chemother 2003;47(1):106–17.
[16] Yustes C, Guarro J. In vitro synergistic interaction between amphotericin B and micafungin against Scedosporium spp. Antimicrob Agents Chemother 2005;49(8):3498–500.
[17] Heyn K, Tredup A, Salvenmoser S, Müller F-MC. Effect of voriconazole combined with micafungin against Candida, Aspergillus, and Scedosporium spp. and *Fusarium solani*. Antimicrob Agents Chemother 2005;49(12):5157–9.
[18] Bhat SV, Paterson DL, Rinaldi MG, Veldkamp PJ. *Scedosporium prolificans* brain abscess in a patient with chronic granulomatous disease: successful combination therapy with voriconazole and terbinafine. Scand J Infect Dis 2007;39(1):87–90.
[19] Howden B, Slavin M, Schwarer A, Mijch A. Successful control of disseminated *Scedosporium prolificans* infection with a combination of voriconazole and terbinafine. Eur J Clin Microbiol Infect Dis 2003;22(2):111–3.
[20] Whyte M, Irving H, O'Regan P, Nissen M, Siebert D, Labrom R. Disseminated *Scedosporium prolificans* infection and survival of a child with acute lymphoblastic leukemia. Pediatr Infect Dis J 2005;24(4):375–7.

Appendix 7 Gastrointestinal granuloma due to *Candida albicans* in an immunocompetent cat

7.1 Introduction

The following appendix contains a publication by Duchaussoy et al., 2015 (http://dx.doi.org/10.1016/j.mmcr.2015.12.002). This publication details a clinical case of focal intestinal candidiasis in a cat (domestic shorthair) that presented to the University of Melbourne Veterinary Teaching Hospital for diagnosis and treatment.

I performed the molecular identification on the clinical samples, identifying the agent of disease as *Candida albicans*, and assisted in drafting and finalising the manuscript.

7.2 Main article

Medical Mycology Case Reports 10 (2015) 14–17

Contents lists available at ScienceDirect

Medical Mycology Case Reports

journal homepage: www.elsevier.com/locate/mmcr

Gastrointestinal granuloma due to *Candida albicans* in an immunocompetent cat

Anne-Claire Duchaussoy [a], Annie Rose [a], Jessica J. Talbot [b], Vanessa R. Barrs [b,*]

[a] University of Melbourne, Veterinary Teaching Hospital 250 Princes Highway, Werribee 3030, Australia
[b] Faculty of Veterinary Science, Valentine Charlton Cat Centre, University of Sydney, NSW 2006, Australia

ARTICLE INFO

Article history:
Received 25 November 2015
Received in revised form
7 December 2015
Accepted 11 December 2015
Available online 12 December 2015

Keywords:
Cat
Gastrointestinal
Fungal
Candida albicans

ABSTRACT

A 3.5 year-old cat was admitted to the University of Melbourne Veterinary Teaching Hospital for chronic vomiting. Abdominal ultrasonography revealed a focal, circumferential thickening of the wall of the duodenum extending from the pylorus aborally for 3 cm, and an enlarged gastric lymph node. Cytology of fine-needle aspirates of the intestinal mass and lymph node revealed an eosinophilic inflammatory infiltrate and numerous extracellular septate acute angle branching fungal-type hyphae. Occasional hyphae had globose terminal ends, as well as round to oval blastospores and germ tubes. *Candida albicans* was cultured from a surgical biopsy of the duodenal mass. No underlying host immunodeficiencies were identified. Passage of an abrasive intestinal foreign body was suspected to have caused intestinal mucosal damage resulting in focal intestinal candidiasis. The cat was treated with a short course of oral itraconazole and all clinical signs resolved.

1. Introduction

There are more than 150 species of Candida but only a small percentage of these are frequent pathogens of humans. *Candida* spp. are commensals of humans and animals and normally inhabit the alimentary, upper respiratory and lower urogenital tracts. Complications secondary to local overgrowth and extension due to impairment of the mucosal barrier are the most common causes of invasive mycoses reported in people but have rarely been described in cats and dogs [1,2].

The ability of *Candida* to adhere to vaginal, gastrointestinal and oral epithelial cells is well documented [2]. However, to become pathogenic, interruption of normal defence mechanisms is necessary. In cats, this can occur as a result of naturally occurring disease including diabetes mellitus, feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) infection, or from iatrogenic factors, especially therapeutic modalities such as antibiotic administration, surgery and indwelling urinary catheters [1]. Most reported cases of candidiasis in companion animals have involved the urinary tract of immunocompromised patients [3,4]. Peritonitis and visceral organ invasion has also been reported in dogs [1]. Here we report the first case of a gastrointestinal granuloma due to *Candida albicans* in a young immunocompetent cat.

2. Case

A 3.5 year-old domestic shorthair cat was referred to the University of Melbourne's Veterinary Teaching Hospital (Day 0) for investigation of chronic intermittent vomiting of 6 months duration. Vomiting occurred 2–3 h after eating every 2–3 days. Appetite was maintained and there was no weight loss. A dietary trial with a hypoallergenic diet (Hill's Z/d dry cat food) did not improve the clinical signs. No antibiotics had been prescribed and anti-inflammatory or intestinal worming treatments had not been administered in the previous 6 months. Deterioration with gradual increased frequency of vomiting and lethargy occurred before presentation to the teaching hospital.

Physical examination revealed a weight of 4.8 kg (body score condition 3/9), normothermia and normal respiratory and cardiovascular auscultation. A small firm mass was palpable in the cranial abdomen. No pain response was elicited on palpation. Fundoscopic examination was unremarkable. A complete blood count showed a mild peripheral eosinophilia (3.7×10^9/L, reference interval [RI] 0–1.5×10^9/L) and normal haematocrit (37%, [RI] 30–45%) and total white cell count (12.8×10^9/L, RI 5–19.5×10^9/L). Results of a serum biochemistry panel were within reference intervals. Serology for detection of FeLV antigens and FIV antibodies was negative (*FASTest*® FeLV-FIV, Diagnostik MEGACOR Germany).

On abdominal ultrasonography there was circumferential

* Corresponding author.

http://dx.doi.org/10.1016/j.mmcr.2015.12.002

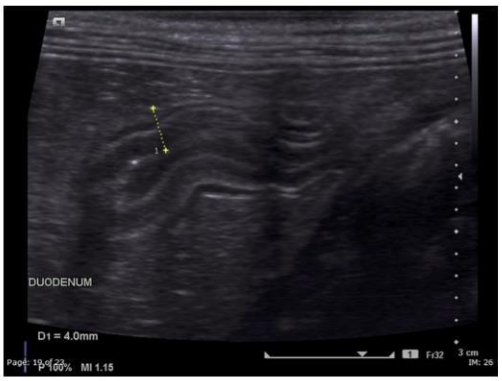


Fig. 1. Abdominal ultrasonographic image showing the focal, circumferential thickening of the descending duodenum.

thickening of the descending duodenum (wall thickness 4.5 mm RI < 3 mm) extending from the pylorus aborally for 3 cm. The muscularis layer was disproportionately thickened, and there was no loss of integrity of normal intestinal wall layering (Fig. 1). The regional gastric lymph node was markedly enlarged (14.9 mm × 8.1 mm), hypoechoic and rounded. The rest of the gastrointestinal tract appeared normal and no other abnormalities were identified.

Ultrasound-guided fine needle aspirates were collected from the thickened region of duodenal wall and enlarged gastric lymph node. On cytology of the gastric lymph node there was an inflammatory infiltrate consisting of abundant eosinophils and non-degenerate neutrophils and occasional well granulated mast cells and macrophages. Lymphoid cells comprised small lymphocytes (54–80%), intermediate lymphocytes (12–34%), large lymphocytes (4–11%) and plasma cells (1–4%). The smears of the duodenal mass were poorly cellular and mainly comprised eosinophils. Differential diagnosis included neoplasia, focal sterile or infectious granuloma (e.g. fungal, protozoal, bacterial or viral including feline infectious peritonitis), feline gastrointestinal eosinophilic sclerosing firbroplasia (FGESF) and an embedded foreign body.

Thoracic radiographs were unremarkable, and an exploratory laparotomy was performed on Day 1. The origin of the proximal duodenum circumferential mass was confirmed to be just cranial to the pyloric sphincter extending 3 cm along the proximal duodenum. The mass was firm and hyperaemic and incorporated the opening of the common bile duct into the duodenum (Fig. 2). Gastric and mesenteric lymph nodes were also enlarged. Full resection of the mass and cholecystojejunostomy was recommended

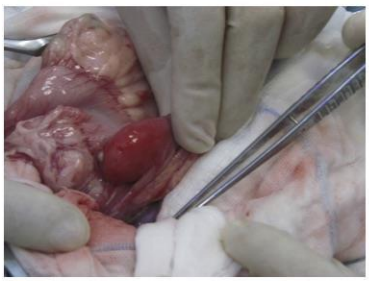


Fig. 2. Focal circumferential lesion in the proximal duodenum visualised during the exploratory laparotomy.

but declined by the owner due to financial constraints and potential complications of the procedure. Fine needle aspirates of the duodenal mass and gastric lymph node, full-thickness incisional biopsies of the mass, stomach, jejunum and ileum and excisional biopsies of the jejunal and gastric lymph nodes were performed. Repeat cytology of the duodenal mass revealed an eosinophil- rich inflammatory response and intralesional fungal elements (Fig. 3). Inflammatory cells comprised neutrophils (32–90%), eosinophils (2–44%), mast cells (0–18%), lymphocytes (6%), macrophages (0–2%) and occasional plasma cells (Fig. 1). In some of the cytological preparations there were numerous extracellular septate acute angle branching fungal hyphae (3–5 μm diameter), occasionally with a globose terminal end. Round to oval blastospores and germ tubes (2–3 μm diameter), some of which were intracellular within neutrophils, were also noted. Repeated cytology of the lymph node confirmed reactive lymphoid hyperplasia.

Histopathological features of the stomach and small intestine were assessed according to World Small Animal Veterinary Association criteria, and were normal [5], except for a mild increase in eosinophils within the lamina propria in the proximal portion of the ileum. There was marked chronic lymphoid hyperplasia of the gastric lymph node with a moderate to marked expansion of the parafollicular lymphocytes. Sinuses contained a small to moderate number of neutrophils and lymphocytes. In the mesenteric lymph node follicles were small with prominent germinal centres. One macrophage within medullary sinuses contained two small round thin-walled structures, suspected to be fungal elements.

On histopathology of the duodenal mass, the submucosa was markedly expanded by irregular connective tissue, fibroblasts, endothelium, and multifocal to coalescing infiltrates of macrophages, neutrophils, lymphocytes and plasma cells, consistent with moderate to marked, and chronic, focally extensive, pyogranulomatous enteritis involving the sub-mucosa. There was evidence of thickening of duodenal muscle layers, as noted on ultrasonography. This was attributed mainly to oedema and regional myofibre degeneration along with subserosal accumulations of eosinophils, neutrophils occasional macrophages and lymphocytes. Further fungal elements could not be identified on H&E or periodic acid Schiff stains on the excisional biopsy sample. Culture of the duodenal mass yielded a pure culture of narrow-necked ovoid, budding, non-capsulate yeasts, which produced germ tubes within 3 h of incubation in serum broth at 35 °C. A panfungal PCR amplification of the ITS1-5.8s-ITS2 region of ribosomal DNA was performed. On comparative sequence analysis there was 100% homology with reference strains of *C. albicans* (GenBank KP131671.1, KP 131658.1) (Irinyi, Serena et al. 2015). Antifungal susceptibility testing was performed at the National Mycology Reference Centre, Adelaide (Table 1).

Fungal and bacterial urine cultures were negative. Post-operative recovery was unremarkable and the cat was treated with itraconazole (5 mg/kg q 12 h orally). Due to poor patient compliance treatment was stopped after 6 days. No further vomiting occurred and appetite and demeanor remained normal.

At a recheck examination 7 weeks after the surgery, the cat was well and had increased in body weight from 4.8 to 5.2 kg. Physical examination and ocular examination including fundoscopy was unremarkable. On repeat haematology there was normalisation of the previous eosinophilia, and serum biochemistry and urinalysis were within RI. Repeat abdominal ultrasonography revealed persistence of a relative thickening of the muscularis layer at the level of the pyloro-dudodenal junction (1.3 mm thick, RI 0.05 mm [6]) for a segment of 8 mm length (previously 30 mm). This change occupied portions of the circumference of the proximal descending duodenum, whereas previously the changes were circumferential. The gastric lymph node was not present; the hepatic lymph node was visualised and was of normal size.

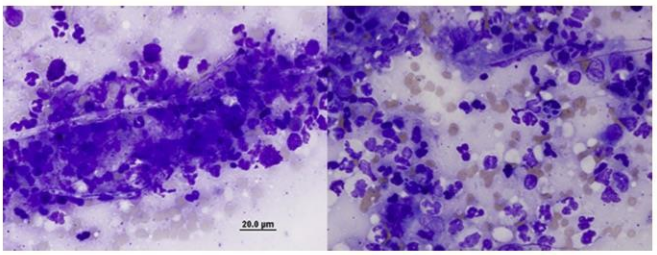


Fig. 3. Cytological preparation of the duodenal mass, modified Wright–Giemsa stain. Note the presence of visible hyphae (A) and round to oval blastospores and germ tubes of 2–3 µm (B).

Table 1
Antifungal susceptibility results for the intestinal *C. albicans* isolate.

Antifungal	MIC (mg/L)
Amphotericin B	0.25 N
5-Fluorocytosine	< 0.06 N
Fluconazole	0.5 S
Itraconazole	0.06 N
Voriconazole	< 0.008 S
Posaconazole	0.015 N
Caspofungin	0.06 S
Anidulafungin	0.06 S
Micafungin	0.015 S

MIC = Minimum inhibitory concentration
S = Sensitive, R = Resistant, I = Intermediate, SDD = Susceptible dose dependent
N = No interpretive criteria are available as yet for this species/drug.

The cat represented 7 months after surgery for 3 days of vomiting. Physical examination was unremarkable and the cat had not lost weight. On repeat abdominal ultrasonography the hepatic lymph node was of normal size and the duodenal lesion was unchanged from the last ultrasound. The previous granuloma was not considered the likely cause for the recent clinical signs. The cat responded to a hypoallergenic diet and improved after a day in hospital. No further vomiting was reported by the owners 9 months after surgery.

3. Discussion

This is the first report of gastro-intestinal candidiasis in a companion animal that is not associated with septic peritonitis secondary to a perforating duodenal ulcer or intestinal dehiscence after surgery [1]. The presence of a single pyogranulomatous intestinal lesion is unusual [7]. No underlying reason could be identified to explain an abnormal or disrupted gastrointestinal intestinal mucosal barrier leading to opportunistic candidiasis. Previously described underlying causes leading to the classical pathway of *Candida* invasion were not present [8]; there was no history of antibiotic, anti-acid, glucocorticoid or anti-inflammatory treatment [7]. Furthermore, in humans, the stomach and the duodenum have a physiologically more acidic environment and the lowest number of *C. albicans* compared to other regions of the gastrointestinal tract. This is likely to be similar in cats and dogs, but has not been confirmed. Hence the stomach and duodenum are unusual sites for candidiasis due to an abnormal or disrupted gastrointestinal microbiome [9]. An abrasive foreign body causing focal mucosal disruption was considered the most likely explanation in this case, even though no foreign material was detected on histopathological examination of multiple sections of the intestinal mass. However, a foreign-body could have caused traumatic intestinal injury during passage without being retained.

Other possible, but less likely explanations for candidiasis in this case include a transient defective local immunity associated with a viral infection (e.g. Coronavirus, Rotavirus) and/or intestinal dysbiosis. Both pathogenic factors and an impaired intestinal mucosal barrier are required for invasive *C. albicans* infection. Virulence factors of *C. albicans* include the ability to adhere to epithelial cells and to form biofilms. The ability to form hyphae is also a virulence factor and is associated with active infection, since hyphal forms can invade epithelial cells and cause tissue damage [10]. In the case here, hyphae were visualised on cytological preparations of the intestinal lesion. This finding, in combination with culture and ITS-sequencing results is evidence of an active infection of *C. albicans*. No bacterial involvement was identified.

Several mechanisms are implicated in the development of *C. albicans* infection, including an inadequate host immune response (Yan, Yang et al. 2013). Induction of the initial mucosal immune responses occurs in the gut associated lymphoid tissue (GALT), within Peyer's patches and mesenteric lymph nodes [11]. Many host cellular pattern recognition receptors (PPARgamma and Dectin-1) have also been identified to interact with *C. albicans* [12,13]. Presence of bile acids and an abnormally increased gastric pH may promote *C. albicans* virulence [14]. Hence chronic gastroenteritis or long term treatment with anti-acids can lead to invasive candidiasis. *C albicans* itself can delay gastrointestinal ulcer healing by releasing cytokines (IL-1beta and TNF-alpha) which perpetuate ongoing inflammation and promote conditions for gastrointestinal wall invasion and adherence [15]. Altered host/*C. albicans* interactions could lead to local and systemic infection. In our cat, no underlying disease, gastro-intestinal bacterial overgrowth, chronic inflammation or systemic debilitating diseases were identified. An improvement 6 weeks after the introduction of a hypoallergenic diet (Hills' Z/d) raises suspicion of a possible inflammatory disorder, which could have been the source of an altered host/*C. albicans* interaction, although the focal nature of the disease makes this less likely.

Recommended treatment for this active yeast infection includes surgical resection followed by medical treatment. In this case, due to financial constraints and poor patient compliance surgical resection was not possible and only a short oral course of itraconazole was administered. However, a dramatic improvement in both clinical signs and the size of the initial lesion was observed 8 weeks post-surgical biopsy (Day 60) and was sustained 9 months post-surgery (Day 275).

Conflict of interest

There are none.

Ethical form

Please note that this journal requires full disclosure of all sources of funding and potential conflicts of interest. The journal also requires a declaration that the author(s) have obtained written and signed consent to publish the case report from the patient or legal guardian(s).

The statements on funding, conflict of interest and consent need to be submitted via our Ethical Form that can be downloaded from the submission site www.ees.elsevier.com/mmcr. Please note that your manuscript will not be considered for publication until the signed Ethical Form has been received.

Acknowledgements

The authors thank Dr. Erin Bell from University of Melbourne Veterinary Teaching Hospital for her help in case management.

References

[1] K. Bradford, J. Meinkoth, K. McKeirnen, B. Love, Candida peritonitis in dogs: report of 5 cases, Veter. Clin. Pathol./Am. Soc. Veter. Clin. Pathol. 42 (2) (2013) 227–233, Epub 2013/06/05.
[2] M.J. Kennedy, P.A. Volz, C.A. Edwards, R.J. Yancey, Mechanisms of association of *Candida albicans* with intestinal mucosa, J. Med. Microbiol. 24 (4) (1987) 333–341, Epub 1987/12/01.
[3] B.M. Pressler, S.L. Vaden, I.F. Lane, L.D. Cowgill, J.A. Dye, Candida spp. urinary tract infections in 13 dogs and seven cats: predisposing factors, treatment, and outcome, J. Am. Anim. Hosp. Assoc. 39 (3) (2003) 263–270, Epub 2003/05/21.
[4] Y. Jin, D.L. Fungal, urinary tract infections in the dogs and cat: a retrospective study (2001–2004), J. Am. Anim. Hosp. Assoc. 41 (6) (2005) 373–381.
[5] M.J. Day, T. Bilzer, J. Mansell, B. Wilcock, E.J. Hall, A. Jergens, et al., Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group, J. Comp. Pathol. 138 (Suppl 1) (2008) S1–43. Epub 2008/03/14.
[6] M.D. Winter, L. Londono, C.R. Berry, J.A. Hernandez, Ultrasonographic evaluation of relative gastrointestinal layer thickness in cats without clinical evidence of gastrointestinal tract disease, J. Feline Med. Surg. 16 (2) (2014) 118–124, Epub 2013/08/03.
[7] L. Yan, C. Yang, J. Tang, Disruption of the intestinal mucosal barrier in *Candida albicans* infections, Microbiol. Res. 168 (7) (2013) 389–395, Epub 2013/04/03.
[8] J.C. Heseltine, D.L. Panciera, G.K. Saunders, Systemic candidiasis in a dog, J. Am. Veter. Med. Assoc. 223 (6) (2003) 821–824 10. Epub 2003/09/26.
[9] S.J. O'Flaherty, T.R. Klaenhammer, Functional and phenotypic characterization of a protein from Lactobacillus acidophilus involved in cell morphology, stress tolerance and adherence to intestinal cells, Microbiology 156 (Pt 11) (2010) 3360–3367, Epub 2010/09/11.
[10] P.E. Sudbery, Growth of *Candida albicans* hyphae, Nat. Rev. Microbiol. 9 (10) (2011) 737–748, Epub 2011/08/17.
[11] D. Luongo, R. D'Arienzo, P. Bergamo, F. Maurano, M. Rossi, Immunomodulation of gut-associated lymphoid tissue: current perspectives, Int. Rev. Immunol. 28 (6) (2009) 446–464, Epub 2009/12/04.
[12] L. Lefevre, A. Gales, D. Olagnier, J. Bernad, L. Perez, R. Burcelin, et al., PPAR-gamma ligands switched high fat diet-induced macrophage M2b polarization toward M2a thereby improving intestinal *Candida* elimination, Plos. one 5 (9) (2010) e12828, Epub 2010/09/30.
[13] A. Gales, A. Conduche, J. Bernad, L. Lefevre, D. Olagnier, M. Beraud, et al., PPARgamma controls Dectin-1 expression required for host antifungal defense against *Candida albicans*, Plos. Pathog. 6 (1) (2010) e1000714, Epub 2010/01/12.
[14] M. Nagi, K. Tanabe, Y. Takano, K. Kikuchi, Y. Miyazaki, M. Niimi, Serum or bile affects the in vitro azole susceptibilities of *Candida* spp, Jpn. J. Infect. Dis. 62 (4) (2009) 306–308, Epub 2009/07/25.
[15] T. Brzozowski, M. Zwolinska-Wcislo, P.C. Konturek, S. Kwiecien, D. Drozdowicz, S.J. Konturek, et al., Influence of gastric colonization with *Candida albicans* on ulcer healing in rats: effect of ranitidine, aspirin and probiotic therapy, Scand. J. Gastroenterol. 40 (3) (2005) 286–296, Epub 2005/06/04.

Appendix 8: Detection of *Aspergillus*-specific antibodies by agar gel immunodiffusion and IgG ELISA for diagnosis of feline upper respiratory tract aspergillosis

8.1 Introduction

The following appendix contains a publication by Barrs et al., 2015 (http://dx.doi.org/10.1016/j.tvjl.2014.12.020). This publication investigates the use of *Aspergillus* specific antibodies as a diagnostic tool for feline upper respiratory tract aspergillosis.

I performed molecular identification on fungal cultures grown from some of the new clinical samples included in this study, identifying the agent of disease as *Aspergillus viridinutans* species complex members, and assisted in drafting and finalising the manuscript.

8.2 Main article

The Veterinary Journal 203 (2015) 285–289

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Detection of *Aspergillus*-specific antibodies by agar gel double immunodiffusion and IgG ELISA in feline upper respiratory tract aspergillosis

V.R. Barrs [a,*], B. Ujvari [a], N.K. Dhand [a], I.R. Peters [b], J. Talbot [a], L.R. Johnson [c], F. Billen [d], P. Martin [a], J.A. Beatty [a], K. Belov [a]

[a] Faculty of Veterinary Science, University of Sydney, NSW 2006, Australia
[b] TDDS, Innovation Centre, University of Exeter, Devon, UK
[c] School of Veterinary Medicine, University of California, Davis, CA 95616, USA
[d] Faculty of Veterinary Medicine, University of Liege, Belgium

ARTICLE INFO

Article history:
Accepted 21 December 2014

Keywords::
Aspergillosis
Aspergillus spp
Sino-nasal
Sino-orbital
Feline

ABSTRACT

Feline upper respiratory tract aspergillosis (URTA) is an emerging infectious disease. The aims of this study were: (1) to assess the diagnostic value of detection of *Aspergillus*-specific antibodies using an agar gel double immunodiffusion (AGID) assay and an indirect immunoglobulin G (IgG) ELISA; and (2) to determine if an aspergillin derived from mycelia of *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus flavus* can be used to detect serum antibodies against cryptic *Aspergillus* spp. in *Aspergillus* section *Fumigati*. Sera from cats with URTA (group 1: $n = 21$) and two control groups (group 2: cats with other upper respiratory tract diseases, $n = 25$; group 3: healthy cats and cats with non-respiratory, non-fungal illness, $n = 84$) were tested. Isolates from cats with URTA comprised *A. fumigatus* ($n = 5$), *A. flavus* ($n = 1$) and four cryptic species: *Aspergillus felis* ($n = 12$), *Aspergillus thermomutatus* (*Neosartorya pseudofischeri*, $n = 1$), *Aspergillus lentulus* ($n = 1$) and *Aspergillus udagawae* ($n = 1$).

Brachycephalic purebred cats were significantly more likely to develop URTA than other breeds ($P = 0.013$). The sensitivity (Se) of the AGID was 43% and the specificity (Sp) was 100%. At a cut-off value of 6 ELISA units/mL, the Se of the IgG ELISA was 95.2% and the Sp was 92% and 92.9% for groups 2 and 3 cats, respectively. *Aspergillus*-specific antibodies against all four cryptic species were detected in one or both assays. Assay Se was not associated with species identity. Detection of *Aspergillus*-specific antibodies by IgG ELISA has high Se and Sp for diagnosis of feline URTA.

Introduction

Feline upper respiratory tract aspergillosis (URTA) is increasingly being recognised (Barrs and Talbot, 2014). There are two anatomical forms of disease, sino-nasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA) (Barrs et al., 2012, 2014). A strong association has been identified between the infecting fungal species and the anatomical form of disease; SNA is most commonly caused by *Aspergillus fumigatus*, while *Aspergillus felis*, a recently discovered 'cryptic' species in *Aspergillus* section *Fumigati* (*Aspergillus viridinutans* complex), is the most common cause of SOA (Barrs et al., 2013, 2014; Barrs and Talbot, 2014). So-called cryptic species are indistinguishable on morphological features from *A. fumigatus sensu stricto*.

Similar to SNA in dogs, feline SNA is usually non-invasive, such that fungal hyphae do not penetrate the respiratory mucosa (Whitney et al., 2005); in contrast, in SOA fungal hyphae invade sino-nasal and paranasal tissues. Invasive mycoses typically occur in immunocompromised hosts. However, systemic immunodeficiency has not been detected in most cats with URTA (Barrs et al., 2012), one exception being a cat with feline leukaemia virus (FeLV) infection (Goodall et al., 1984).

The sensitivity (Se) of serological tests for detection of fungal antigens or *Aspergillus*-specific antibodies in aspergillosis depends on the systemic immunocompetence of the host as reflected by the ability to clear fungal antigen from the circulation and to mount an antibody response. An ELISA to detect a fungal cell wall antigen, galactomannan (GM), in serum (Platelia *Aspergillus* EIA, Bio-Rad) has a Se of up to 90% in immunocompromised patients, including neutropenic human patients with pulmonary aspergillosis and dogs with disseminated invasive aspergillosis (DIA) (Maertens et al., 1999; Garcia et al., 2012). However, the Se of this test is <30% in non-neutropenic human patients with aspergillosis, in immunocompetent dogs with SNA and in cats with URTA (Billen et al., 2009; Kitasato et al., 2009; Whitney et al., 2013).

* Corresponding author. Tel.: +61 2 93513437.
E-mail address: vanessa.barrs@sydney.edu.au (V.R. Barrs).

http://dx.doi.org/10.1016/j.tvjl.2014.12.020
1090-0233/

Conversely, detection of serum *Aspergillus*-specific antibodies by agar gel double immunodiffusion (AGID) or by immunoglobulin G (IgG) ELISA has a high test Se in immunocompetent patients, including dogs with SNA (67–88%) and humans with chronic pulmonary aspergillosis (74–94%) (Pomrantz et al., 2007; Billen et al., 2009; Guitard et al., 2012; Ohba et al., 2012). A detectable antibody response is mounted in <30% of neutropenic humans with aspergillosis and dogs with DIA (Day et al., 1985; Hope et al., 2005; Schultz et al., 2008).

We hypothesised that *Aspergillus*-specific antibodies would be detectable in the majority of cats with URTA, since most cats with URTA are not, as far as it is possible to currently evaluate, systemically immunocompromised. The aims of this study were: (1) to assess the diagnostic value of detection of *Aspergillus*-specific antibodies using an AGID assay and an indirect IgG ELISA; and (2) to determine if a commercial aspergillin derived from mycelia of *A. fumigatus, Aspergillus niger* and *Aspergillus flavus* can be used to detect serum antibodies against cryptic *Aspergillus* spp. in *Aspergillus* section *Fumigati*.

Materials and methods

Signalment data and serum (1–2 mL per cat) were collected prospectively from cats diagnosed with URTA (group 1), cats with upper respiratory tract (URT) signs not attributable to aspergillosis (group 2), and from cats without respiratory or fungal disease (group 3). Samples were collected with informed consent according to the guidelines of the Animal Ethics Committee of the University of Sydney (approval number N00/9–2012/5774, date of approval 22 June 2012). Serum samples were collected at the time of diagnosis and were stored at −80 °C for batch testing.

Animals

Group 1: Cats with upper respiratory tract aspergillosis (n = 21)

Inclusion criteria for cats with URTA were a complete medical history, consistent clinical signs, identification of fungal hyphae on cytology and/or histopathology of tissue from the sino-nasal cavity or orbit, and a positive fungal culture (Barrs et al., 2012). Cases with mixed fungal infections were excluded. Isolates were identified using phenotypic features and comparative sequence analyses of the internal transcribed spacer (ITS) regions (ITS1-5.8S-ITS2), partial β-tubulin and/or partial calmodulin genes (Barrs et al., 2013), except for *A. fumigatus* identification, where consistent phenotypic features and demonstration of growth at 50 °C were acceptable alternatives to molecular identification (Barrs and Talbot, 2014). Isolates comprised *A. fumigatus* (*n* = 5), *A. flavus* (*n* = 1) and four cryptic species in *Aspergillus* section *Fumigati*, i.e. *A. felis* (*n* = 12), *Aspergillus thermomutatus* (syn. *Neosartorya pseudofischeri*, *n* = 1), *Aspergillus lentulus* (*n* = 1) and *Aspergillus udagawae* (*n* = 1) (Table 1).

Cats were classified as having SOA (*n* = 12) or SNA (*n* = 9) based on the presence (SOA) or absence (SNA) of a retrobulbar mass on computed tomography (CT) or magnetic resonance imaging (MRI) at diagnosis. Sera were tested for antibodies against feline immunodeficiency virus (FIV) and FeLV antigen (IDEXX SNAP Combo, IDEXX Laboratories). Medical histories were analysed for the presence of comorbidities. All cats were neutered, comprising 11 male neutered (MN) and 10 female neutered (FN) cats, and the median age was 5 years (range 2–14 years). Breeds comprised domestic crossbred (*n* = 8), Persian (*n* = 4), Ragdoll (*n* = 3), Himalayan (*n* = 2), British shorthair (*n* = 1), Scottish shorthair (*n* = 1), Cornish Rex (*n* = 1) and Abyssinian (*n* = 1).

Group 2: Control cats with other URT disease (n = 25)

Inclusion criteria were: (1) consistent clinical signs, e.g. sneezing, nasal discharge; (2) absence of fungal hyphae on cytology or histology of tissue collected from the sino-nasal cavity; and/or (3) serological, histopathological or endoscopic diagnosis of another URT disease. Standard diagnostic investigations included latex antigen cryptococcal serology (CALAS, Meridian Bioscience), upper airway endoscopy, CT examination of the sino-nasal cavity, fungal culture and biopsy. This group included cats with chronic rhinosinusitis (*n* = 9), nasal neoplasia (*n* = 10) (lymphoma, *n* = 4; adenocarcinoma, *n* = 3; squamous cell carcinoma, *n* = 2; osteosarcoma, *n* = 1), upper respiratory cryptococcosis (*n* = 5) and nasopharyngeal stenosis (*n* = 1). All cats were neutered (13 MN, 12 FN). The median age was 11 years (range 4–16 years). Breeds comprised domestic crossbred (*n* = 14), Persian (*n* = 2), Siamese/Oriental (*n* = 2), Russian blue (*n* = 2), Cornish Rex (*n* = 2), British shorthair (*n* = 1), Burmilla (*n* = 1) and Tonkinese (*n* = 1).

Group 3 (i and ii): Control cats without respiratory or fungal disease (n = 84)

Inclusion criteria for group 3 were (i) healthy cats presenting to the Valentine Charlton Cat Centre (VCCC) for neutering, vaccination or wellness examination, and for group 3 were (ii) sick cats presenting to the VCCC for non-fungal, non-respiratory illness. Exclusion criteria for groups 3 (i) and 3 (ii) were any clinical signs within the last 4 weeks or findings at physical examination suggestive of respiratory disease. This group comprised (i) 36 healthy cats, including five male entire (M), 11 MN, six female entire (F) and 14 FN, and (ii) 48 cats presented for non-fungal, non-respiratory illness (one M, 23 MN, 24 FN). Diagnoses in cats with non-respiratory disease included hyperthyroidism or post radio-iodine treatment of hyperthyroidism recheck (*n* = 12), enteropathy e.g. enteritis, intestinal foreign body (*n* = 11), chronic kidney disease (*n* = 8), allergic skin disease (*n* = 4), central nervous system disease (*n* = 3), diabetes mellitus (*n* = 2), pancreatitis (*n* = 2), cholelithiasis (*n* = 1), chyloabdomen (*n* = 1), anaemia (*n* = 1), dog bite wound (*n* = 1), portosystemic shunt (*n* = 1) and idiopathic hypocalcaemia (*n* = 1).

Overall, there were 39 males (six M, 33 MN) and 45 females (six F, 39 FN) in group 3; the median age was 8 years (range 0.7–19.5 years). The median age of group 3 (i) cats was 3 years (range 0.7–12 years) and the median age of group 3 (ii) cats was 12 years (range 1–19.5 years). Breeds comprised domestic crossbred (*n* = 67), Burmese (*n* = 3), Ragdoll (*n* = 3), Devon Rex (*n* = 2) and one each of Cornish Rex,

Table 1
Fungal species and serology results for group 1 cats with upper respiratory tract aspergillosis.

Cat[a]	Age (years)	Sex	Breed	Form	Fungal species [b]	AGID	ELISA (units/mL)
1	2	MN	Ragdoll	SNA	*A. thermomutatus (N. pseudofischeri)*	–	35.7
2	2	FN	DSH	SOA	*A. felis*	+	273.1
3	5	FN	Cornish Rex	SOA	*A. felis*	–	797.9
4	13	MN	DSH	SNA	*A. felis*	–	5
5	14	FN	Persian cross	SNA	*A. lentulus*	–	38
6	3	MN	DSH	SOA	*A. felis*	–	26
7	8	FN	Persian	SOA	*A. felis*	+	215.5
8	2	MN	British shorthair	SOA	*A. felis*	+	110.7
9	7	MN	Persian	SNA	*A. fumigatus*	–	28.2
10	2	MN	Himalayan	SOA	*A. felis*	+	35.8
11	8	MN	DLH	SOA	*A. udagawae*	+	55.7
12	8	FN	Scottish shorthair	SNA	*A. fumigatus*	+	56.6
13	5	FN	DSH	SOA	*A. felis*	+	154.9
14	4	MN	Ragdoll	SOA	*A. felis*	+	49.7
15	3	FN	Himalayan	SOA	*A. felis*	+	78.3
16	2	FN	DSH	SOA	*A. felis*	–	295.1
17	14	FN	Abyssinian	SNA	*A. fumigatus*	–	16.1
18	3	MN	Ragdoll	SOA	*A. felis*	–	288.42
19	14	FN	Persian	SNA	*A. fumigatus*	–	24.6
20	4	MN	DSH	SNA	*A. flavus*	–	6.3
21	7	MN	DSH	SNA	*A. fumigatus*	–	82.4

AGID, agar gel immunodiffusion; DSH, domestic shorthair; DLH, domestic longhair; FN, female neutered; MN, male neutered; SNA, sino-nasal aspergillosis; SOA, sino-orbital aspergillosis.

[a] Country of origin was Australia except cats 17 (USA), 19 (UK) and 21 (Belgium).

[b] Signalment of cat and molecular identity of isolates for cats 1–15 has been reported elsewhere (Barrs et al., 2013, 2014; Barrs and Talbot, 2014).

Abyssinian, Siamese, Birman, Bengal, Persian, Russian blue, British shorthair and Singapura.

Agar gel double immunodiffusion

Detection of precipitating anti-*Aspergillus* antibodies by AGID (Ouchterlony method) was performed using a commercially available test-kit (Fungal Immunodiffusion Kit, Meridian Bioscience) comprising agar immunodiffusion plates, an aspergillin derived from the mycelial phase of cultures of *A. fumigatus, A. niger* and *A. flavus* with a protein content of 1486 μg/mL (Aspergillus Immunodiffusion Antigen reference number 100501, Meridian Bioscience) and goat anti-*Aspergillus* immunodiffusion control serum (reference number 100901, Meridian Bioscience). Testing was performed in accordance with the manufacturer's instructions using 20 μL each of control sera, test serum and aspergillin. All samples were tested in duplicate. Gels were examined for the presence of precipitin bands of identity or partial identity after 24 h and again after an additional 48 h incubation (final reading) in a humidified chamber at room temperature. Visualisation of precipitin bands was facilitated by directing a high-intensity light beam at a 45° angle below the plate, with the latter held against a black background.

Aspergillus-specific IgG quantification by indirect ELISA

An indirect ELISA for detection and quantification of *Aspergillus*-specific IgG antibodies in canine sera using the same aspergillin as for the AGID was modified for use in cats (Billen et al., 2009). Binding activity using polyvinylchloride or polystyrene 96-well plates was assessed as similar. Two commercially available secondary antibodies, rabbit anti-cat IgG (H&L), ALP conjugated, were evaluated (SAB 37008-1, Sigma; AS10 1479, Agrisera).

The assay was optimised by performing checkerboard titrations to determine the optimal dilutions of antigen, cat serum and secondary antibody. Inter- and intra-plate coefficients of variation were calculated by running 40 repeats of the positive control sample (pooled positive control sera from seven cats with confirmed aspergillosis and a positive AGID result) on four separate plates with 10 repeats on each plate. Test samples were run in duplicate and each plate contained a duplicate positive control, a negative control (pooled negative control sera from 15 healthy controls with a negative AGID result), and a blank (phosphate buffered saline, PBS, plus 0.05% Tween 20, Sigma; PBS-T). Sera were titrated in doubling dilutions from 1:800 to 1:102,400.

Ninety-six well enzyme immunoassay (EIA)/radioimmunoassay (RIA) polystyrene plates (Costar 3590, Corning) were coated with 75 μL aspergillin (2.5 μg protein/mL) and incubated at 4 °C overnight. Plates were blocked with 75 μL 1% w/v polyvinylpyrrolidone (Sigma) in PBS for 1 h at room temperature. Fifty microlitres of patient sera was diluted in 5% non-fat milk in PBS-T, titrated on plates in doubling dilutions from 1:800 to 1:102,400 and incubated for 2 h at 37 °C. Fifty microlitres of 1:8000 rabbit anti-cat IgG (H&L), ALP conjugated, antibody solution (SAB 37008-1, Sigma) diluted in PBS-T was added to each well and incubated for 1 h at 37 °C. Next, 200 μL Sigma*FAST* p-nitrophenyl alkaline phosphate substrate (Sigma) was added to each well, incubated in the dark for 45 min then stopped with 50 μL of 3 M NaOH. Optical density (OD) was determined using a plate reader with a 405 nm and 492 nm wavelength filter (Benchmark Plus microplate spectrophotometer, Bio-Rad Laboratories). All incubations were performed in a humidified chamber and wells were washed three times between incubations with 150 μL PBS.

Statistical analysis

The mean age of cats was compared between groups using a general linear model. Sex proportions were compared using a χ^2 test. For the purpose of statistical analyses, breeds were grouped into brachycephalic (Persian/Persian-cross, Himalayan, Ragdoll, Birman, Burmilla, British/Scottish shorthair) and non-brachycephalic (Domestic short/longhair, Cornish/Devon Rex, Bengal, Russian blue, Oriental, Siamese, Tonkinese, Singapura). Proportions of cats in brachycephalic and non-brachycephalic groups, and proportions of positive test results for cats infected with *A. fumigatus* versus cryptic species were compared using Fisher's exact test.

Values for median ELISA units (EU) in group 1 were compared between AGID positive and AGID negative cats, and between cats with *A. fumigatus* infections and those infected with cryptic species, using non-parametric Mann and Whitney *U* tests. For analysis of ELISA data, the geometric mean optical OD for each set of duplicate serum samples was calculated and $\log_{10}$ OD values were plotted against $\log_{10}$ serum dilutions for positive control and test sera in Microsoft Excel. The curves generated were compared for parallelism and IgG concentrations were expressed as EU/mL, with the positive control serum standard having a concentration of 100 EU/mL (Billen et al., 2009). Serum samples with fewer than three dilution points within the linear range of the standard, and thus considered to have antibody concentrations below the detectable limit of the ELISA (<2.5 EU/mL), were assigned a value of 0 EU/mL. An association between age and IgG quantification in EU/mL in controls was investigated using simple linear regression.

Cut-off values were established by determination of the mean plus three SD of the IgG concentration of the controls and by receiver operating characteristic (ROC) analysis. ROC analysis was conducted by fitting a logistic regression model of log EU values on the binary outcome (1 or 0) created by specifying the URTA group as 1 and the control group as 0 (Dohoo et al., 2009). ROC analyses were conducted for group 2, group 3 and both groups combined. The optimal cut-off value for each analysis was determined using Youden's J index. Se and specificity (Sp) at the determined cut-off values were reported as described by de Silva et al. (2013). Analyses were conducted using SAS 2002–2003 (SAS Institute/IBM). A 5% level of significance was used for all statistical tests.

Results

Cats

In group 1, one cat (cat 5) was determined to be FIV-infected on the basis of a positive FIV antibody response and no history of FIV vaccination; the other 20 cats in group 1 tested negative for FIV and FeLV (Table 1). The mean age of cats in group 1 (6.3 years) was significantly different from that of cats in group 2 (9.8 years; $P<0.01$) and group 3 (i) (4.0 years, $P<0.01$), but not from the combined group 3 (i and ii: 8.2 years, $P=0.1$) or a combined control group (groups 2 + 3: 8.7 years; $P=0.06$). There were no significant differences in sex between groups ($P=0.9$). The proportions of brachycephalic breeds were significantly different between groups 1 and 2, and between groups 1 and 3, but not between groups 2 and 3 ($P=0.2$); 11/21 (52%) group 1 cats were brachycephalic compared to 4/25 (16%) group 2 cats ($P<0.05$) and 4/84 (5%) group 3 cats ($P<0.01$).

Agar gel double immunodiffusion

Nine of 21 sera (43%) from cats with URTA (group 1) were positive in the AGID (Table 1). Sera from all 25 cats in group 2 and 84 cats in group 3 tested negative. The Se, Sp, positive predictive value (PPV) and negative predictive value (NPV) of AGID for the diagnosis of URTA are given in Table 2. Of the nine cats with positive AGID results, one was infected with *A. fumigatus* and eight were infected with cryptic species, including *A. felis* ($n=7$) and *A. udagawae* ($n=1$) (Table 1). There was no association between test result (positive or negative) and infecting species (*A. fumigatus* versus cryptic species; $P=0.3$).

IgG ELISA

The intra- and inter-plate coefficients of variation of the ELISA were 7.4% and 9.3%, respectively. Sera from cats that did not generate a dilution curve with a minimum of three dilution points within the range of the standard serum were assigned an *Aspergillus*-specific IgG concentration of 0 EU/mL (20/25 group 2 cats and 74/84 group 3 cats). Cut-off values calculated using the mean plus three SD of the IgG concentration and using ROC analysis were similar, yielding results of 5.6 and 6.0 EU/mL, respectively, regardless of the control group used. Se and Sp were optimal at a cut-off value of 6 EU/mL

Table 2
Diagnostic accuracy of agar-gel double immunodiffusion in 21 cats with sino-nasal and sino-orbital aspergillosis.

	Control group 2[a] ($n=25$)		Control group 3[b] ($n=84$)		Control groups 2 and 3 ($n=109$)	
	%	95% CI	%	95% CI	%	95% CI
Se	42.9	21.9–66.0	42.9	21.9–66.0	42.9	21.9–66.0
Sp	100.0	86.2–100.0	100.0	95.7–100.0	100.0	96.6–100.0
PPV	100.0	66.2–100.0	100.0	66.2–100.0	100.0	66.2–100.0
NPV	87.5	50.2–100.0	97.7	79.2–93.4	90.1	83.3–94.8

CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

[a] Cats with other upper respiratory tract diseases (excluding aspergillosis).

[b] Healthy controls ($n=36$) and sick cats ($n=48$) with non-fungal, non-respiratory illness.

Table 3
Performance of IgG ELISA for detection of *Aspergillus*-specific antibodies at different cut-off values.

Controls	AUC	95% CI for AUC	Cut-off value (EU/mL)					
			5		6		9	
			Se	Sp	Se	Sp	Se	Sp
Group 2	0.97	0.92–1.00	100.0% (21/21)	92.0% (23/25)	95.2% (20/21)	92.0% (23/25)	90.5% (19/21)	96.0% (24/25)
Group 3	0.97	0.98–1.00	100.0% (21/21)	91.7% (77/84)	95.2% (20/21)	92.9% (78/84)	90.5% (19/21)	100.0% (84/84)
Group 2 and 3	0.99	0.97–1.00	100.0% (21/21)	91.7% (100/109)	95.2% (20/21)	92.7% (101/109)	90.5% (19/21)	99.1% (108/109)

CI, confidence interval; AUC, area under curve; EU, ELISA Units; SE, sensitivity; SP, specificity.

(Table 3); at this cut-off value, the Se was 95.2%, the Se was 92.0%, the PPV was 90.9% (95% confidence interval, CI, 70.8–98.6%) and the NPV was 95.5% (95% CI 78.8–99.3%).

Using the calculated cut-off value of 6.0 EU/mL, a positive IgG ELISA result was obtained for sera from 20/21 (95.2%) cats with URTA (range 6.3–797.9 EU/mL) (Table 1), from 2/25 (8.0%) cats, both with cryptococcal rhinitis, in group 2 (8.7 and 80.7 EU/mL) and 6/84 (7.1%) cats in group 3 (7.3–8.9 EU/mL) (Fig. 1). The median *Aspergillus*-specific IgG concentration in cats with URTA (group 1) was 55.7 EU/mL. Among cats with URTA, there was no significant difference in *Aspergillus*-specific IgG concentrations in cases with *A. fumigatus* infection (median 67 EU/mL; $n = 5$) and cases with infection by cryptic species (other members of the *A. fumigatus* complex; median 56.6 EU/mL; $n = 15$; $P = 0.1$). There was no association between test result (positive or negative) and infecting species (*A. fumigatus* versus cryptic species; $P = 0.3$). The median *Aspergillus*-specific IgG concentrations of cats with positive AGID results was 78.3 EU/mL, compared to 31.95 EU/mL for cats with negative AGID results ($P = 0.2$). There was no significant effect of age on EU values of combined groups 2 and 3 ($P = 0.05$) or group 3 alone ($P = 0.2$).

Discussion

In this study, we demonstrated that antibodies against four cryptic species of *Aspergillus* (*A. felis*, *A. udagawae*, *A. lentulus* and *A. thermomutatus*) can be detected in feline serum with assays utilising a commercial aspergillin derived from *A. fumigatus*, *A. niger* and *A. flavus*. Although this result was not unexpected given the close phylogenetic relationship of these cryptic species to *A. fumigatus* (Barrs et al., 2013; Novakova et al., 2014), it is important to demonstrate this cross reactivity, given the high frequency of infections with such cryptic species in cats. *A. felis* and *A. udagawae* are the two most commonly reported species of *Aspergillus* to cause SOA in cats (Kano et al., 2008, 2013; Barrs et al., 2013, 2014; Barrs and Talbot, 2014).

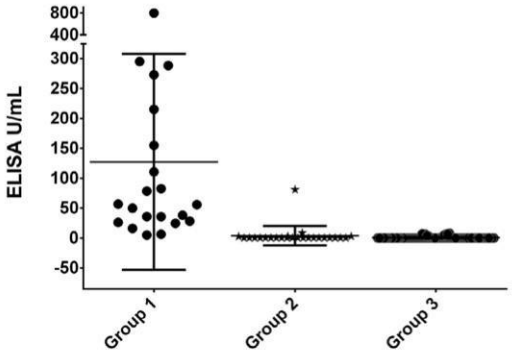


Fig. 1. ELISA units/mL for 21 group 1 sera (cats with aspergillosis; black circles), 25 group 2 sera (control cats with other upper respiratory tract disease; black stars) and 84 group 3 sera (control cats, either healthy or sick with non-respiratory disease; grey circles). Lines represent means ± standard deviations.

There was a marked difference in the Se of the AGID and the IgG ELISA for detection of *Aspergillus*-specific antibodies, while the Sp for both assays was high. In contrast to the IgG ELISA, which detects one class of antibody, immunodiffusion assays detect precipitins (Crowle, 1973). In AGID assays, optimal diffusion depends on many factors including sufficiently large antigen (Ag) and antibody (Ab) reservoirs to maintain infinite pools of reactants (Kunkel, 1988). Since a commercial test kit was utilised in the present study, optimisation of the assay was not performed. The same commercial AGID has been evaluated for diagnosis of canine SNA, which is caused by *A. fumigatus* in >95% of cases, with reported Se of 57–67% (Pomrantz et al., 2007; Pomrantz and Johnson, 2010; Barrs and Talbot, 2014). Another commercial AGID (Immuno-Mycologics) had a Se of 31% for diagnosis of canine SNA (Peeters and Clercx, 2007). The highest reported Se of 76.5% using an AGID for diagnosis of canine SNA utilised a customised Ouchterlony method (Billen et al., 2009).

The IgG ELISA had high Se and Sp overall, indicating that the production of *Aspergillus*-specific IgG is a reliable indicator of URTA. Depending on the cut-off value and control group used, the Se of the assay was 91–100% and the Sp was 92–100%. Cases with URT diseases other than aspergillosis (group 2) represent the most relevant control group in a clinical situation. Of interest, both cats with false positive IgG results in group 2 had cryptococcosis. The high antibody titre detected in one cat with sino-orbital cryptococcosis (80.7 EU/mL) was repeatable. The cat had a latex cryptococcal antigen titre of 1024 (Meridian, CALAS 2010) and *Cryptococcus gattii* was cultured from the nasal cavity. On CT there was a retrobulbar mass arising from the nasal cavity, but yeasts were not seen on cytology of fine-needle aspirate biopsies. The cat was treated with itraconazole for one year until the LCAT decreased to zero and clinical signs resolved. Possible explanations for the high antibody titre are a false positive disease result or a true result due to co-infection with *Aspergillus* spp., which is possible, but unlikely. Concurrent pulmonary cryptococcosis and aspergillosis has been documented rarely in humans (Lin et al., 2006; Enoki et al., 2012).

The high frequency of *Aspergillus*-specific IgG and the low frequency of retroviral infection by serology in group 1 cats (0% for FeLV; 4.7% for FIV) provide further evidence that cats with URTA are not systemically immunocompromised (Whitney et al., 2013). Other causes of immunosuppression documented in cats with DIA, such as feline panleukopenia, feline infectious peritonitis or prolonged corticosteroid therapy (Ossent, 1987), were not evident among cats with URTA tested in the present study. However, local disease that may have predisposed to sino-nasal cavity fungal colonisation was identified in two cats; one cat with *A. fumigatus* infection had concurrent nasal adenocarcinoma (cat 17) and one cat with *A. flavus* infection had plant material removed from the nasal cavity during endoscopy (cat 20) (Table 1). To further our understanding of the immunopathogenesis of this disease, additional studies of the humoral response to URTA, including quantification of IgM and IgA in affected cats, are warranted.

Our finding that purebred cats of brachycephalic conformation were significantly more likely to develop URTA confirms a predisposition which, until now, has only been suspected (Tomsa et al., 2003; Whitney et al., 2005; Barrs et al., 2012). Impaired sinus aeration and drainage associated with brachycephalic skull conformation that favours fungal colonisation have been proposed as a mechanism for this breed association (Tomsa et al., 2003). A heritable disorder of innate immunity has also been proposed (Barrs and Talbot, 2014). Chronic invasive granulomatous fungal rhinosinusitis of humans, similar to feline SOA, occurs in immunocompetent people in the Indian subcontinent, especially those working in agriculture and construction (Thompson and Patterson, 2012). In contrast to feline SOA, the aetiological agent is usually *A. flavus*. *A. flavus* is an uncommon cause of URTA in cats and only a single case has been identified previously (Malik et al., 2004).

Using a cut-off value of 5 EU/mL to optimise the IgG ELISA for Se makes this assay an ideal screening test for URTA in cats with consistent clinical signs; positive results should be corroborated with additional tests, such as CT/MRI findings, rhinosinuscopy, cytological or histological detection of fungal elements in affected tissues and fungal culture. Assay Sp was not 100% even at the cut-off value optimised for Sp (9 EU/mL) in group 2 cats, the most clinically relevant control group. Therefore, serology should not be relied upon as the sole diagnostic test for URTA.

Conclusion

Detection of *Aspergillus*-specific IgG by AGID and ELISA was highly specific for the diagnosis of aspergillosis in cats. The Se of IgG detection by ELISA was high, whereas the Se of detection using AGID was low. Depending on the cut-off value used, the ELISA has good discriminatory power to distinguish between presumed environmental exposure, which increases with age, and that induced by colonisation and infection. This study provides further evidence that feline URTA affects systemically immunocompetent individuals.

Conflict of interest statement

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This study was funded by an Australian Companion Animal Health Foundation grant (015/2013). The sponsors were not involved in any aspect of the study or in the decision to publish this manuscript. The authors thank many colleagues for contributions of clinical samples for this study.

References

Barrs, V.R., Talbot, J., 2014. Feline aspergillosis. Veterinary Clinics of North America: Small Animal Practice 44, 51–73.

Barrs, V.R., Halliday, C., Martin, P., Wilson, B., Krockenberger, M., Gunew, M., Bennett, S., Koehlmeyer, E., Thompson, A., Fliegner, R., et al., 2012. Sinonasal and sino-orbital aspergillosis in 23 cats: Aetiology, clinicopathological features and treatment outcomes. The Veterinary Journal 191, 58–64.

Barrs, V.R., van Doorn, T., Houbraken, J., Kidd, S.E., Martin, P., Pinheiro, M.D., Richardson, M., Varga, J., Samson, R.A., 2013. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats and dogs. PLoS ONE 8, e64781.

Barrs, V.R., Beatty, J.A., Dhand, N., Talbot, J., Bell, E., Abraham, L.A., Chapman, P., Bennett, S., van Doorn, T., Makara, M., 2014. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. The Veterinary Journal 201, 215–222.

Billen, F., Peeters, D., Peters, I.R., Helps, C.R., Huynen, P., De Mol, P., Massart, L., Day, M.J., Clercx, C., 2009. Comparison of the value of measurement of serum galactomannan and *Aspergillus*-specific antibodies in the diagnosis of canine sino-nasal aspergillosis. Veterinary Microbiology 133, 358–365.

Crowle, A.J., 1973. Immunodiffusion, Second Ed. Academic Press, New York, NY, USA, pp. 1–78.

de Silva, K., Purdie, A.C., Kawaji, S., Dhand, N.K., Whittington, R.J., 2013. Can early host responses to mycobacterial infection predict eventual disease outcomes? Preventive Veterinary Medicine 112, 203–212.

Day, M.J., Eger, C.E., Shaw, S.E., Penhale, W.J., 1985. Immunological study of systemic aspergillosis in German shepherd dogs. Veterinary Immunology Immunopathology 9, 335–347.

Dohoo, I., Martin, W., Stryhn, H., 2009. Veterinary Epidemiologic Research, second Ed. VER, Charlottetown, Prince Edward Island, Canada. 865 pp.

Enoki, E., Maenishi, O., Chikugo, T., Ito, A., Kimura, M., 2012. Coinfection of *Aspergillus* and *Cryptococcus* in post-tuberculosis pulmonary cavity. Pathology International 62, 574–576.

Garcia, R.S., Wheat, L.J., Cook, A.K., Kirsch, E.J., Sykes, J.E., 2012. Sensitivity and specificity of a blood and urine galactomannan antigen assay for diagnosis of systemic aspergillosis in dogs. Journal of Veterinary Internal Medicine 26, 911–919.

Goodall, S.A., Lane, J.G., Warnock, D.W., 1984. The diagnosis and treatment of a case of nasal aspergillosis in a cat. Journal of Small Animal Practice 25, 627–633.

Guitard, J., Sendid, B., Thorez, S., Gits, M., Hennequin, C., 2012. Evaluation of a recombinant antigen-based enzyme immunoassay for the diagnosis of noninvasive aspergillosis. Journal of Clinical Microbiology 50, 762–765.

Hope, W.W., Walsh, T.J., Denning, D.W., 2005. Laboratory diagnosis of invasive aspergillosis. Lancet Infectious Diseases 5, 609–622.

Kano, R., Itamoto, K., Okuda, M., Inokuma, H., Hasegawa, A., Balajee, S.A., 2008. Isolation of *Aspergillus udagawae* from a fatal case of feline orbital aspergillosis. Mycoses 51, 360–361.

Kano, R., Shibahashi, A., Fujino, Y., Sakai, H., Mori, T., Tsujimoto, H., Yanai, T., Hasegawa, A., 2013. Two cases of feline orbital aspergillosis due to *A. udagawae* and *A. virdinutans*. Journal of Veterinary Medical Science 75, 7–10.

Kitasato, Y., Tao, Y., Hoshino, T., Tachibana, K., Inoshima, N., Yoshida, M., Takata, S., Okabayashi, K., Kawasaki, M., Iwanaga, T., et al., 2009. Comparison of *Aspergillus* galactomannan antigen testing with a new cut-off index and *Aspergillus* precipitating antibody testing for the diagnosis of chronic pulmonary aspergillosis. Respirology (Carlton, Vic.) 14, 701–708.

Kunkel, J.G., 1988. Analytical immunologic techniques. In: Gilbert, L.I., Miller, T.A. (Eds.), Immunological Techniques in Insect Biology. Springer, New York, NY, USA, pp. 1–41.

Lin, C.M., Tsai, Y.H., Huang, C.C., Lee, C.H., Chiang, P.C., Huang, S.F., Liu, H.P., 2006. Invasive pulmonary aspergillosis and pulmonary cryptococcosis really coexist in immunocompromised host. Journal of Infection 53, e55–e58.

Maertens, J., Verhaegen, J., Demuynck, H., Brock, P., Verhoef, G., Vandenberghe, P., van Eldere, J., Verbist, L., Boogaerts, M., 1999. Autopsy-controlled prospective evaluation of serial screening for circulating galactomannan by a sandwich enzyme-linked immunosorbent assay for hematological patients at risk for invasive aspergillosis. Journal of Clinical Microbiology 37, 3223–3228.

Malik, R., Vogelnest, L., O'Brien, C.R., White, J., Hawke, C., Wigney, D.I., Martin, P., Norris, J.M., 2004. Infections and some other conditions affecting the skin and subcutis of the naso-ocular region of cats – clinical experience 1987–2003. Journal of Feline Medicine and Surgery 6, 383–390.

Novakova, A., Hubka, V., Dudova, Z., Matsuzawa, T., Kubatova, A., Yaguchi, T., Kolarik, M., 2014. New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex. Fungal Diversity 64, 253–274.

Ohba, H., Miwa, S., Shirai, M., Kanai, M., Eifuku, T., Suda, T., Hayakawa, H., Chida, K., 2012. Clinical characteristics and prognosis of chronic pulmonary aspergillosis. Respiratory Medicine 106, 724–729.

Ossent, P., 1987. Systemic aspergillosis and mucormycosis in 23 cats. Veterinary Record 120, 330–333.

Peeters, D., Clercx, C., 2007. Update on canine sinonasal aspergillosis. Veterinary Clinics of North America: Small Animal Practice 37, 901–916.

Pomrantz, J.S., Johnson, L.R., 2010. Repeated rhinoscopic and serologic assessment of the effectiveness of intranasally administered clotrimazole for the treatment of nasal aspergillosis in dogs. Journal of the American Veterinary Medical Association 236, 757–762.

Pomrantz, J.S., Johnson, L.R., Nelson, R.W., Wisner, E.R., 2007. Comparison of serologic evaluation via agar gel immunodiffusion and fungal culture of tissue for diagnosis of nasal aspergillosis in dogs. Journal of the American Veterinary Medical Association 230, 1319–1323.

Schultz, R.M., Johnson, E.G., Wisner, E.R., Brown, N.A., Byrne, B.A., Sykes, J.E., 2008. Clinicopathologic and diagnostic imaging characteristics of systemic aspergillosis in 30 dogs. Journal of Veterinary Internal Medicine 22, 851–859.

Thompson, G.R., Patterson, T.F., 2012. Fungal disease of the nose and paranasal sinuses. Journal of Allergy and Clinical Immunology 129, 321–326.

Tomsa, K., Glaus, T.M., Zimmer, C., Greene, C.E., 2003. Fungal rhinitis and sinusitis in three cats. Journal of the American Veterinary Medical Association 222, 1380–1384.

Whitney, B.L., Broussard, J., Stefanacci, J.D., 2005. Four cats with fungal rhinitis. Journal of Feline Medicine and Surgery 7, 53–58.

Whitney, J., Beatty, J.A., Dhand, N., Briscoe, K., Barrs, V.R., 2013. Evaluation of serum galactomannan detection for the diagnosis of feline upper respiratory tract aspergillosis. Veterinary Microbiology 162, 180–185.

Appendix 9: Sinonasal aspergillosis in a British shorthair cat in the United Kingdom

9.1 Introduction

The following appendix contains a publication by Tamborini et al., 2016 (https://doi.org/10.1177/2055116916653775). This publication details a clinical case of sino-nasal aspergillosis in a cat (British shorthair) that presented to the VRCC, Laindon, UK for diagnosis and treatment. This is the first reported case of SNA in a cat in the United Kingdom.

I performed the molecular identification on the clinical samples, identifying the agent of disease as *Aspergillus fumigatus*, and assisted in drafting and finalising the manuscript.

9.2 Main article

Case Report

jfms OPEN REPORTS

Journal of Feline Medicine and Surgery Open Reports
1–6

DOI: 10.1177/2055116916653775
jfmsopenreports.com

This paper was handled and processed by the European Editorial Office (ISFM) for publication in JFMS Open Reports

SAGE

Sinonasal aspergillosis in a British Shorthair cat in the UK

Alice Tamborini[1], Elise Robertson[1], Jessica J Talbot[2] and Vanessa R Barrs[2]

Abstract

Case summary A 13-year-old, castrated male, British Shorthair cat presented for investigation of chronic, intermittent, bilateral epistaxis and stertor. CT revealed severe asymmetric bilateral intranasal involvement with extensive turbinate lysis, increased soft tissue attenuation and lysis of the sphenopalatine bone and cribriform plate. On retroflexed pharyngoscopy, a plaque-like mass occluded the choanae. Rostral rhinoscopic examination revealed extensive loss of nasal turbinates, necrotic tissue and mucosal fungal plaques in the left nasal cavity. The right nasal cavity was less severely affected. The nasal cavities were debrided extensively of plaques and necrotic tissue. *Aspergillus fumigatus* was isolated on fungal culture, and species identity was confirmed using comparative sequence analysis of the partial β-tubulin gene. On histopathology of nasal biopsies, there was ulcerative lymphoplasmacytic and neutrophilic rhinitis, and fungal hyphae were identified on nasal mucosa, consistent with a non-invasive mycosis. The cat was treated with oral itraconazole after endoscopic debridement, but signs relapsed 4.5 months from diagnosis. Residual left nasal fungal plaques were again debrided endoscopically and oral posaconazole was administered for 6 months. Fourteen months from diagnosis, the cat remains clinically well with mild intermittent left nasal discharge secondary to atrophic rhinitis.

Relevance and novel information This is the first case of rhinoscopically confirmed sinonasal aspergillosis to be diagnosed in a cat in the UK. Endoscopic confirmation of resolution of infection is useful in cases where mild nasal discharge persists after treatment.

Accepted: 16 May 2016

Introduction

Aspergillosis is a mycotic disease affecting a diverse range of human and animal hosts, including mammals and birds.[1–7] In cats, two forms of aspergillosis affect the upper respiratory tract: sinonasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA). In dogs, SNA accounts for >99% of reported cases and is considered non-invasive. This is in contrast to feline aspergillosis, where SOA is reported to be the most common form (65% of reported cases) and is invasive.[1–7]

Case description

A 13-year-old, castrated male, British Shorthair cat presented for investigation of chronic, intermittent, bilateral mucopurulent nasal discharge, epistaxis, stertor and anorexia. The cat had been diagnosed with lymphoplasmacytic rhinitis elsewhere, 5 years previously. On physical examination, no abnormalities were found and vital parameters were normal. Results of a complete blood count, coagulation profile and biochemistry were within normal limits. CT of the skull revealed extensive abnormalities affecting both nasal cavities, including bilateral obliteration of the air spaces of the nasal meati extending from the nares rostrally, to the ethmoid ethmoid turbinates caudally. Turbinate detail was obscured by soft tissue/fluid attenuating material, with extension of

[1]VRCC, Laindon, UK
[2]Faculty of Veterinary Science, School of Life and Environmental Sciences, University of Sydney, Sydney, NSW 2006 Australia

Corresponding author:
Vanessa Barrs BVSc, PhD, MVetClinStud, FANZCVS, Faculty of Veterinary Science, University of Sydney, Sydney, NSW 2006, Australia
Email: vanessa.barrs@sydney.edu.au

similarly attenuating material into the nasopharynx. There was destruction of the sphenopalatine bone on the left side. A focal area of increased attenuation of the right nasal turbinates was also evident at the level of the rostral orbit, but there was no mass effect or erosion/displacement of the vomer bone. The cribriform plate was not intact. No changes were detected in the orbital, frontal and nasal bones.

A 4.0 mm × 60 cm bronchoscope was used for retroflexed pharyngoscopy under general anaesthesia. A large 3 cm × 2 cm plaque was visualised occluding the caudal nasopharynx. A bilateral infraorbital splash nerve block was administered (bupivacaine HCl BP 2.64 mg/ml equivalent to bupivacaine HCl anhydrous 2.5 mg/ml, 0.5 mg/kg total) prior to performing rostral rhinoscopy. The caudal oropharynx was packed with swabs to protect the lower airways. Rostral lavage of the nasal cavity was performed using 10 ml aliquots of sterile saline but failed to dislodge material for diagnostic sampling.

Nasal cavity examination was performed using a 1.9 mm × 30 degree oblique integrated telescope (Karl Storz 67030BA) with continuous saline irrigation. There was extensive turbinate bone destruction in both nasal cavities, mainly involving the middle nasal meati to the level of the ethmoid turbinates, and the ventrocaudal meatus. Fungal plaques were observed adherent to nasal mucosa and surrounded by polypoid tissue (Figure 1). The left nasal cavity was more severely affected than the right. Both nasal cavities were aggressively debrided of fungal plaques using 3 mm cupped biopsy forceps (Karl Storz 69133) at premeasured depths following confirmation of plaque locations on rhinoscopic evaluation (Figure 1). Fresh oropharyngeal swabs were pre-placed and 10 ml aliquots of saline were injected via the rhinoscope egress port for rostrocaudal evacuation of plaques. Post-debridement rostral rhinoscopic examination was performed to confirm complete removal of all visible fungal plaques. The oropharynx was actively suctioned throughout the procedure and on recovery to prevent aspiration. A 0.1% ephedrine nasal solution was applied topically into the nasal cavities using cotton swabs inserted into the nostrils, to stem postoperative haemorrhage. An oesophageal feeding tube was placed.

On histopathology of the nasal biopsies there was moderate-to-marked ulcerative lymphoplasmacytic and neutrophilic rhinitis, and fungal hyphae were identified. There was no evidence of invasion of the submucosa by fungal hyphae, with the organisms accumulating on the mucosal surface. Histology of the polypoid tissue observed on rhinoscopy showed hyperplastic, reactive tissue. *Aspergillus fumigatus* was isolated from culture of plaque material, and definitively identified on comparative sequence analysis of the internal transcribed spacer (ITS) region and partial β-tubulin genes and alignment with reference sequences of *A fumigatus* (100% identity).

Other supportive therapy included intravenous fluid therapy (Hartmann's solution 2 ml/kg/h; Aquapharm, Animalcare UK) at 2 ml/kg/h, buprenorphine (Buprecare 0.015 mg/kg q6–8h IM; Animalcare UK), pradofloxacin (Veraflox 25 mg/ml oral suspension 7.5 mg/kg q24h PO; Bayer US), meloxicam (Metacam 0.05 mg/kg q24h PO; Boehringer Ingelheim Vetmedica US) and itraconazole (Itrafungol 10 mg/ml oral solution, 1.5 mg/kg q24h PO; Elanco UK). The oesophageal feeding tube was removed at recheck examination 2 weeks after discharge from hospital due to marked clinical improvement including return of appetite. Renal parameters and liver enzyme activities were within reference intervals (RIs) on serum biochemistry. Meloxicam was stopped after 5 weeks.

Three months after starting itraconazole treatment, a marked increase in alanine aminotransferase (ALT) concentration (1460 U/l; RI 12–130 U/l) was detected on routine monitoring at a re-check examination and itraconazole was withdrawn. Pradofloxacin was continued and a combination of S-adenosylmethionine (SAMe; 20 mg/kg q24h PO) and silybin (Zentonil Advanced 5 mg/kg q24h PO; Vetoquinol France) was prescribed for 2 weeks. Two weeks later, the cat re-presented for recurrence of clinical signs (mucoid nasal discharge, epistaxis and stertor). ALT concentration (176 U/l) was mildly increased. Itraconazole oral suspension was restarted at a lower dose (1 mg/kg q 24h PO). Pradofloxacin and a combination of SAMe and silybin were continued.

Despite itraconazole therapy, the nasal discharge progressively worsened, ALT concentration again increased (301 U/l) and the cat became inappetent. Six months after initial diagnosis, a repeated bilateral rostral rhinoscopic evaluation was performed. Fungal plaques were identified in the left nasal cavity at the level of the ethmoid turbinates in the middle nasal meatus (Figure 2). Aggressive rhinoscopic debridement was performed, as previously described. After the procedure, posaconazole (Noxafil 40 mg/ml oral suspension; Merck Sharp & Dohme) commenced at 2.5 mg/kg q12h PO with food, for 6 months. Pradofloxacin, SAMe and silybin were stopped once clinical improvement and normal ALT concentration were obtained, respectively. Clinical signs resolved and liver enzymes remained normal during therapy.

Four weeks after stopping posaconazole, the cat re-presented with mild nasal discharge. Rhinoscopic evaluation was performed to rule out possible clinical relapse. There was extensive turbinate atrophy on the left side allowing direct visualisation into the left frontal sinus. Areas of polypoid tissue involving the dorsal and middle meati were also seen (Figure 3).The right nasal cavity was similarly but less severely affected. No fungal plaques were observed. The nasal discharge was considered likely secondary to the atrophic rhinitis, although biopsy was not performed. The cat was discharged with no medications and an improvement of the nasal

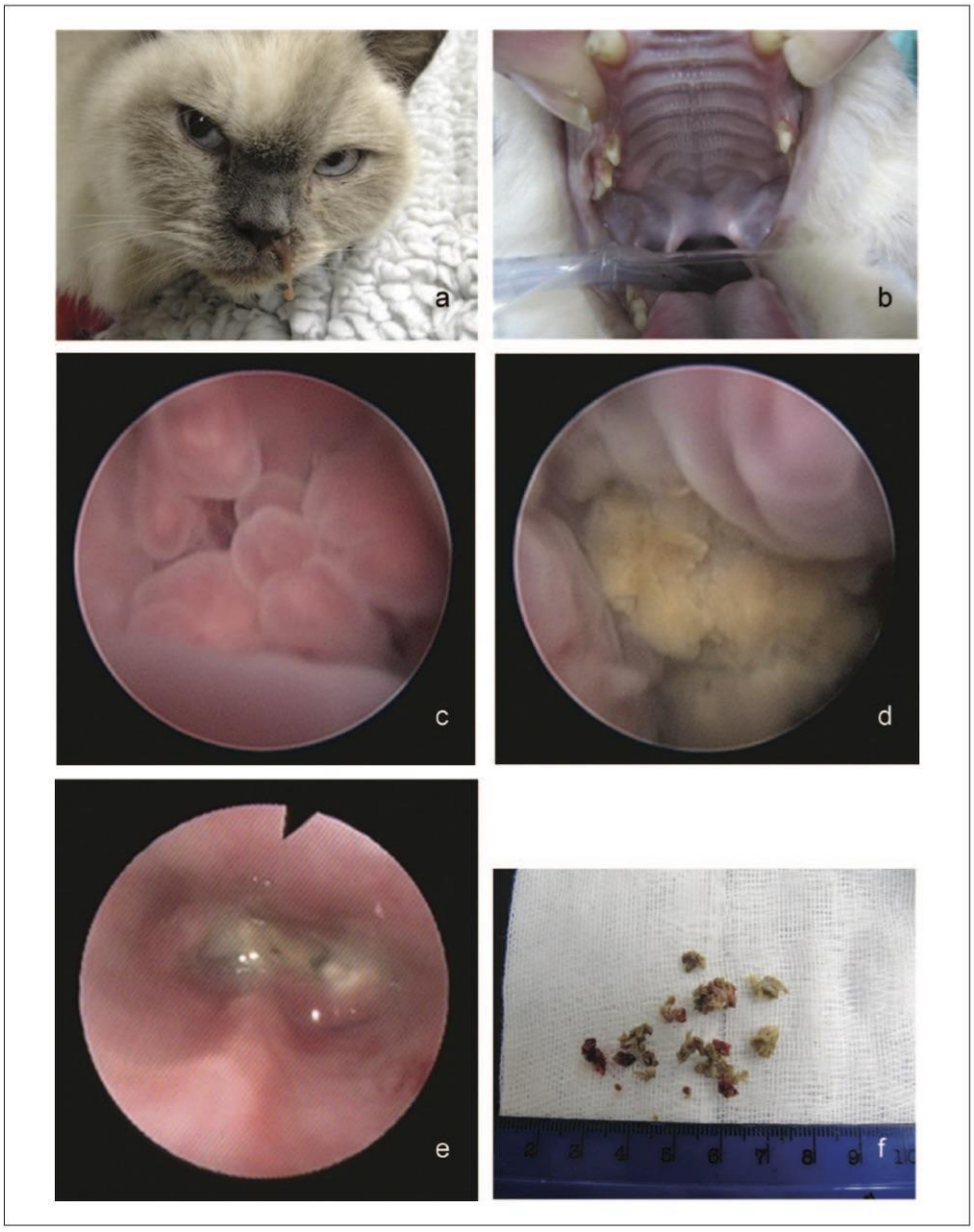


Figure 1 British Shorthair cat with sinonasal aspergillosis at presentation: (a) haemopurulent nasal discharge; (b) oral examination, note the absence of a mass or ulceration in the pterygopalatine fossa commonly seen in sino-orbital aspergillosis; (c) anterograde rhinoscopy left nasal meatus, polypoid and hyperplastic appearance of nasal mucosa in the left nasal meatus; (d) anterograde rhinoscopy left nasal meatus, fungal plaques adherent to nasal mucosa; (e) retroflexed pharyngoscopy, fungal plaques visualised in the choanae; (f) fungal plaques removed with active debridement

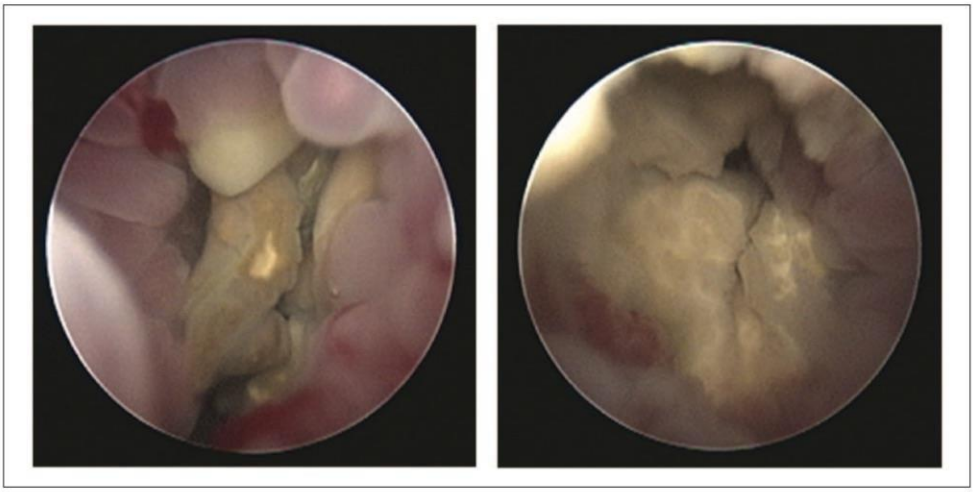


Figure 2 Anterograde rhinoscopy. Left nasal meatus 6 months after diagnosis: fungal plaques and polypoid appearance of nasal mucosa, confirming relapse of infection

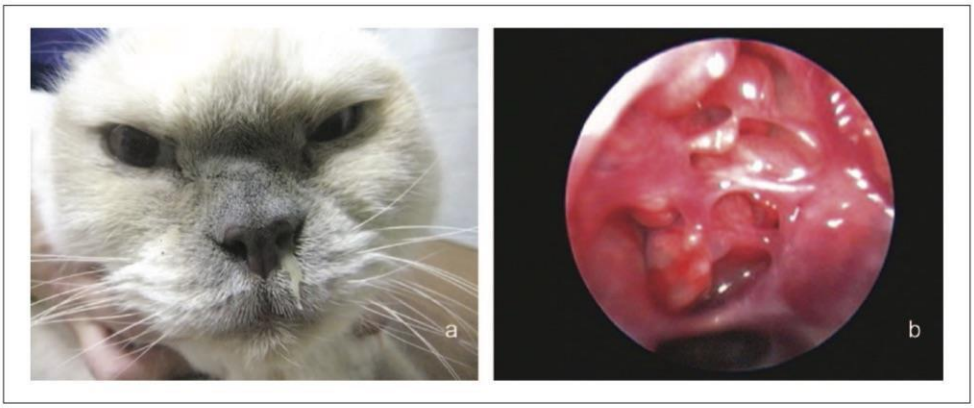


Figure 3 Twelve months after diagnosis: (a) mucopurulent nasal discharge; (b) anterograde rhinoscopy showing atrophic rhinitis and sinus exposure

discharge was seen. Thirteen months from initial diagnosis, the cat remains clinically well.

Discussion

Feline upper respiratory tract aspergillosis (URTA) has been rarely reported in Europe.[1–7] To our knowledge, only eight other cases of URTA have been described in cats from Belgium, Italy, Switzerland and the UK.[1–7] SNA and SOA are the most common forms of aspergillosis in European dogs and cats, respectively.[1–17] The breed of cat in this report reflects the previously reported predisposition of pure-bred brachycephalic cats of Persian lineage to develop URTA.[6] No sex predilection is apparent and pure-bred brachycephalic cats account for more than a third of all cases. The median age at diagnosis is reported to be 6.5 years (range 16 months–13 years).[1–17]

The exact pathogenesis of SNA remains unclear. However, sinonasal mucosal colonisation associated with reduced mucociliary clearance, decreased number and/or function of phagocytic cells, or impaired sinus

aeration and drainage has been described in humans.[18–22] In general, cats with SNA and SOA are systemically immunocompetent. Whether the predisposition for aspergillosis in brachycephalic pure-bred cats, as in this case, is due to conformational abnormalities of the skull, affecting nasolacrimal drainage or an inherited defect in fungal immunity.[2,6,11] Nasal polyposis is a risk factor for fungal rhinosinusitis in humans, but the polypoid changes observed in the nasal mucosa of this cat were considered most likely to be a reactive change to fungal infection.[22]

Clinical signs of SNA in cats are similar to those reported for chronic rhinosinusitis, including sneezing, nasal discharge and epistaxis.[1,3–12,16,17] In the present case, despite evidence of cribriform plate lysis on the CT images, no neurological signs were observed. This reflects our experience, in which cribriform plate destruction is reasonably common in canine and feline SNA, but fungal invasion into the central nervous system (CNS) is rare. This is in contrast to feline SOA, where CNS involvement is more common, especially in chronic infections.[6]

Definitive diagnosis in this case was based on identification of fungal plaques during rhinoscopy, and fungal hyphae on histopathology of nasal biopsies, along with positive fungal culture.[1,6,15] Comparative sequence analysis of the ITS and partial β-tubulin genes confirmed the molecular identity of the infecting isolate in this cat as *A fumigatus*, the most commonly reported cause of SNA in cats.[6,7] Where molecular identification facilities are not readily available, *A fumigatus* can be differentiated from other species in *Aspergillus* section *fumigati* by its ability to grow at 50°C.[6]

Treatment of SNA includes debridement of fungal lesions along with systemic antifungal therapy or topical intranasal azole infusion (clotrimazole or enilconazole), or a combination of all three.[1,2,11,15,23] Debridement of fungal plaques is key to successful resolution of canine and feline SNA. In this case, the cat was treated with aggressive rhinoscopic debridement followed by oral itraconazole. Intranasal clotrimazole infusion was not performed due to the breach in the cribriform plate. Intranasal azole infusion is generally contraindicated in this situation as fatal meningoencephalitis can occur if clotrimazole contacts the brain.[5,24,25] In this case it was considered that treatment benefits were negated by potential significant adverse effects. Unfortunately, when systemic itraconazole treatment was suspended because of hepatotoxicity, relapse of infection occurred. Based on clinical outcome in previous cases of SNA and reported adverse neurological effects to voriconazole, we decided to use posaconazole as a second-line antifungal treatment.[4,11,16,26–28] Posaconazole is well tolerated after oral administration and is infrequently associated with hepatotoxicity.[11,16,26] Given the good initial response of the case here to itraconazole, the lack of success after restarting itraconazole at the time of clinical relapse may have been due to the lack of fungal plaque debridement prior to re-starting the treatment. Posaconazole offers a good second-line monotherapy treatment and has a low risk of hepatotoxicity. In the light of the emergence of azole-resistant isolates of *A fumigatus* in the environment and among clinical strains in people and dogs, antifungal susceptibility testing, which was not performed in this case, is recommended.

Conclusions

To our knowledge, this is the first case of rhinoscopically confirmed SNA diagnosed in a cat living in the UK. Endoscopic debridement of fungal plaques was an important treatment modality. Topical intranasal infusion of clotrimazole was not performed because of a breach in the cribriform plate. Posaconazole monotherapy was useful for long-term management after hepatotoxicity occurred with itraconazole monotherapy.

Funding The authors received no financial support for the research, authorship, and/or publication of this article.

Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

1 Goodall SA, Lane JG and Warnock DW. **The diagnosis and treatment of a case of nasal aspergillosis in a cat**. *J Small Anim Pract* 1984; 25: 627–633.
2 Tomsa K, Glaus TA, Zimmer C, et al. **Fungal rhinitis and sinusitis in three cats**. *J Am Vet Med Assoc* 2003; 222: 1380–1384.
3 Declercq J, Declercq L and Fincioen S. **Unilateral sino-orbital and subcutaneous aspergillosis in a cat**. *Vlaams Diergeneeskd Tijdschr* 2012; 81: 357–362.
4 Barachetti L, Mortellaro CM, Di Giancamillo M, et al. **Bilateral orbital and nasal aspergillosis in a cat**. *Vet Ophthalmol* 2009; 12: 176–182.
5 Nelson RW and Couto CG. **Disorders of nasal cavity: nasal mycosis**. In: Small animal internal medicine. 4th ed. St Louis, Missouri, USA: Elsevier, 2009, 226–229.
6 Barrs VR and Talbot JJ. **Feline aspergillosis**. *Vet Clin North Am Small Anim Pract* 2014; 44: 51–73.
7 Barrs VR, van Doorn TM, Houbraken J, et al. ***Aspergillus felis* sp nov, an emerging agent of invasive aspergillosis in humans, cats, and dogs**. *PLOS ONE* 2013; 8: e64871.
8 Wilkinson GT, Sutton RH and Grono LR. ***Aspergillus spp* infection associated with orbital cellulitis and sinusitis in a cat**. *J Small Anim Pract* 1982; 23: 127–131.
9 Davies C and Troy GC. **Deep mycotic infections in cats**. *J Am Anim Hosp Assoc* 1996; 32 : 380–391.
10 Hamilton HL, Whitley RD and McLaughlin SA. **Exophthalmos secondary to aspergillosis in a cat**. *J Am Anim Assoc* 2000; 36: 343–347.

11 Barrs VR, Halliday C, Martin P, et al. **Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes**. *Vet J* 2012: 191: 58–64.

12 Kano R, Shibahashi A, Fujino Y, et al. **Two cases of feline orbital aspergillosis due to *A udagawae* and *A virdinutans***. *J Vet Med Sci* 2013; 75: 7–10.

13 Malik R, Vogelnest L, O'Brien CR, et al. **Infectious and some other conditions affecting the skin and subcutis of the naso-ocular region of cats: clinical experience 1987–2003**. *J Feline Med Surg* 2004; 6 : 383–390.

14 Karnik K, Reichle JK, Fischetti AJ, et al. **Computed tomographic findings of fungal rhinitis and sinusitis in cats**. *Vet Radiol Ultrasound* 2009; 50: 65–68.

15 Furrow E and Groman RP. **Intranasal infusion of clotrimazole for the treatment of nasal aspergillosis in two cats**. *J Am Vet Med Assoc* 2009; 235: 1188–1193.

16 Giordano C, Gianella P, Bo S, et al. **Invasive mould infections of the naso-orbital region of cats: a case involving *Aspergillus fumigatus* and an etiological review**. *J Feline Med Surg* 2010; 12: 714–723.

17 Smith LN and Hoffman SB. **A case series of unilateral orbital aspergillosis in three cats and treatment with voriconazole**. *Vet Ophthalmol* 2010; 13: 190–203.

18 Segal BH. **Medical progress aspergillosis**. *N Engl J Med* 2009; 360: 1870–1884.

19 Shoham S and Levitz SM. **The immune response to fungal infections**. *Br J Haematol* 2005; 129: 569–582.

20 Romani L. **Immunity to fungal infections**. *Nat Rev Immunol* 2011; 11: 275–288.

21 Latgé JP. **The pathobiology of *Aspergillus fumigatus***. *Trends Microbiol* 2001; 9: 382–389.

22 Thompson GR and Patterson TF. **Fungal disease of the nose and paranasal sinuses**. *J Allergy Clin Immunol* 2012; 129: 321–326.

23 Whitney BL, Broussard J and Stefanacci JD. **Four cats with fungal rhinitis**. *J Feline Med Surg* 2005; 7: 53–58.

24 Zonderland JL, Stork CK, Saunders JH, et al. **Intranasal infusion of enilconazole for treatment of sinonasal aspergillosis in dogs**. *J Vet Med Assoc* 2002; 221: 1421–1425.

25 Peeters D and Clercx C. **Update on canine sinonasal aspergillosis**. *Vet Clin North Am Small Anim Pract* 2007; 37: 901–916.

26 McLennan GJ, Aquino SM, Mason DR, et al. **Use of posaconazole in the management of invasive orbital aspergillosis in a cat**. *J Am Anim Hosp Assoc* 2006; 42: 302–307.

27 Quimby JM, Hoffman SB, Duke J, et al. **Adverse neurologic events associated with voriconazole use in 3 cats**. *J Vet Intern Med* 2010; 24: 647–649.

28 Kano R, Itamoto K, Okuda M, et al. **Isolation of *Aspergillus udagawae* from a fatal case of feline orbital aspergillosis**. *Mycoses* 2008; 51: 360–361.