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Feline Sino-nasal and Sino-orbital Aspergillosis

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Doctor of Philosophy

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results, agreed to host my visit to the CBS-KNAW Fungal Biodiversity Centre at Utrecht. Thanks to Dr. Jos Houbraken and Ms. Tineke van Doorn at CBS for helping me to up-skill in molecular mycology and for sharing their knowledge and expertise and to Dr. Janos Varga for assisting with phylogenetic analyses. Thanks also to Dr. Sarah Kidd Head, National Mycology Reference Centre Microbiology & Infectious Diseases, for her expertise in antifungal susceptibility testing, to Dr. Iain Peters for technical advice in interpretation of ELISA data and to Professor Michael Day for hosting my visit to the University of Bristol that will result in future research publications.

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Declaration

I declare that this thesis is a 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 All information derived from published or unpublished work of others has been acknowledged in the text. A reference list is provided at the end of the thesis.

Vanessa R. D. Barrs

30th June 2014

Author contributions

Included in this thesis are manuscripts that have been published or are in press. I am the primary author of all publications. According to the University of Sydney policy on submission of a thesis by publication, a signed statement regarding the contribution of co-authors is submitted for each published work. Below is a list of manuscripts, followed by the co-authorship confirmation statements for each of these. For brevity, copies of these statements signed by each of the co-authors have been submitted to the Faculty of Veterinary Science Administrative Office.

Manuscripts included in chapters of this thesis.

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, clinicopathologic features and treatment outcomes. The Veterinary Journal 19, 58-64.

Barrs, V.R., van Doorn, T.M., Houbraken, J., Kidd, S.E., Martin, P., Pinheiro, M.D., Richardson, R., Varga, J., Samson, R.A, 2013. *Aspergillus felis* sp. nov, an emerging agent of invasive aspergillosis in humans, cats and dogs. Public Library of Science One 8, e64871; http://dx.doi.org/10.1371/journal.pone.0064871

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 [Epub ahead of print Feb 28], http://dx.doi.org/10.1016/j.tvjl.2014.02.020.

Barrs, V.R., Ujvari, B., Dhand, N., Peters, I., 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 [Epub ahead of print 21.12.14] http://dx.doi.org/10.1016/j.tvjl.2014.12.020

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Confirmation of co-authorship of published work

Barrs, V.R., Halliday, C., Martin, P., Wilson, B., Krockenberger, M., Gunew, M., Bennett, S., Koelmeyer, 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, clinicopathologic features and treatment outcomes. The Veterinary Journal 19, 58-64.

Author's contributions

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Vanessa Barrs designed this project, recruited cases and contributed to the primary analysis and writing up of this publication entitled "Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathologic features and treatment outcomes".

Drs. Catriona Halliday and Associate Prof Beatty, assisted in the design of the project, and provided assistance in finalizing the manuscript prior to publication. Dr Halliday and Ms. Sue Sleiman performed ITS PCR and sequencing. Dr Bethany Wilson performed statistical analyses. Dr Patricia Martin and Dr Ailsa Hocking performed fungal cultures and mating tests. Dr Mark Krockenberger carried out histopathologic examinations and provided assistance in finalising the manuscript prior to publication. Drs. Ann Thompson, Marcus Gunew, Susan Bennett, Eloise Koelmeyer, Reuben Fliegner and Carolyn O'Brien contributed clinical case material and provided assistance in finalising the manuscript prior to publication.

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

Name: Signature: Date:

Confirmation of co-authorship of published work

Barrs, V.R., van Doorn, T.M., Houbraken, J., Kidd, S.E., Martin, P., Pinheiro, M.D., Richardson, R., Varga, J., Samson, R.A, 2013. *Aspergillus felis* sp. nov, an emerging agent of invasive aspergillosis in humans, cats and dogs. Public Library of Science One 8, e64871; http://dx.doi.org/10.1371/journal.pone.0064871

Author's contributions

Vanessa Barrs designed this project, provided clinical isolates, carried out DNA extraction, PCR and sequencing, performed temperature growth studies and mating tests and contributed to the primary analysis and writing up of this publication entitled "*Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats and dogs".

Prof Rob Samson, Dr Jos Houbraken and Tineke van Doorn co- designed the project and assisted with phylogenetic analyses. Dr Jos Houbraken also assisted with design of *MAT1* primers. Tineke van Doorn also carried out DNA extraction, PCR and sequencing and assisted with morphological studies. Dr Sarah Kidd performed antifungal susceptibility testing. Dr Janos Varga assisted with phylogenetic analysis and assisted in finalising the manuscript prior to publication. Dr Patricia Martin assisted with morphological studies. Dr Maria Pinheiro and Prof Malcolm Richards provided clinical isolates. Al authors assisted in finalising the manuscript for publication.

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

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Author's contributions

Vanessa Barrs designed this project, performed molecular identification of isolates and contributed to the primary analysis and writing up of this publication entitled "Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis"

Dr. Mariano Makara assisted in design and interpreted computed tomographic images. Dr Navneet Dhand assisted with statistical analyses. Dr. Jessica Talbot and Ms. Tineke van Doorn performed molecular identification of some isolates. Drs. Julia Beatty, Erin Bell, Linda Abraham, Susan Bennett and Peter Chapman contributed clinical case material. All authors assisted in finalising the manuscript for publication.

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

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Barrs, V.R., Talbot, J.J, 2014. Feline aspergillosis. Veterinary Clinics of North America 44, 51-73; http://dx.doi.org/10.1016/j.cvsm.2013.08.001

Author's contributions

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Vanessa Barrs, the candidate wrote the sections of the review on feline upper respiratory aspergillosis. Dr Jessica Talbot wrote the section on disseminated and non-respiratory focal invasive aspergillosis (page 52, page 53) and fungal virulence factors (page 58), and assisted in finalising the manuscript prior to publication.

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

Name: Signature: Date:

Abbreviations

IA	Invasive aspergillosis
IPA	Invasive pulmonary aspergillosis
BenA	Betatubulin
CalM	Calmodulin
CT	Computed tomographic
FeLV	Feline leukaemia virus
FIV	Feline immunodeficiency virus
FS	Frontal sinus
FURTA	Feline upper respiratory tract aspergillosis
ITS	Internal transcribed spacer
MRI	Magnetic resonance imaging
NC	Nasal cavity
NP	Nasopharynx
SNA	Sino-nasal aspergillosis
SNC	Sino-nasal cavity
SOA	Sino-orbital aspergillosis
SS	Sphenoid sinus
URT	Upper respiratory tract
URTA	Upper respiratory tract aspergillosis

Abstract

Aspergillosis, a mycosis caused by fungi from Genus *Aspergillus,* occurs in many different hosts including insects, amphibians, reptiles, birds and mammals. Upper respiratory tract aspergillosis (URTA) is an emerging mycosis of domestic cats worldwide. The disease encompasses two anatomic forms, sino-nasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA). This thesis characterizes the syndrome of URTA in cats including clinical disease and treatment outcomes. Phenotypic, physiologic and phylogenetic analyses resulted in the discovery of a novel species within *Aspergillus* section *Fumigati, A. felis*. Aspects of disease pathogenesis were explored through comparison of features from computed tomographic (CT) images of soft-tissue and bony structures of the head from affected cats with molecular identity of aetiological agents. Evaluation of the specific-*Aspergillus* antibody response in cats with aspergillosis was performed to develop a non-invasive test for rapid diagnosis and shed further light on disease pathogenesis.

Clinicopathological findings, aetiology and treatment outcomes in 23 cats with URTA recruited retrospectively (1998-2006) and prospectively (2007-2009) were documented. Fungal pathogens were identified by PCR and DNA sequencing (ITS1 or ITS1-5.8S-ITS2 regions, rDNA gene cluster). The median age was 5 y (1.5-13y) and no sex bias was identified. SOA (n=17) was three times more common than SNA (n=6). Pure-bred cats with brachycephalic confirmation comprised more than a third of the study population (n=9). Fungal culture was positive in 22/23 cases. *Aspergillus fumigatus* (*n* = 4), *Neosartorya fischeri* or *A. lentulus* (*n* = 1) and a non-speciated *Neosartorya* spp. (*n* = 1) were identified from cats with SNA. In all cases of SOA (*n* = 17), the fungal pathogen was identified as *Neosartorya* spp. A teleomorphic phase was identified in a subset of these isolates, (*n*=14) indicating a single heterothallic species. ITS sequences from these isolates had closest ancestry with several species within the *Aspergillus viridinutans* complex. Cats with SNA were significantly more likely to be infected with *A. fumigatus* and had a better prognosis than cats with SOA.

We describe a novel heterothallic species in *Aspergillus* section *Fumigati,* namely *A. felis* (neosartorya-morph) isolated from three host species with invasive aspergillosis including a human patient with chronic invasive pulmonary aspergillosis, domestic cats with invasive fungal rhinosinusitis and a dog with disseminated invasive aspergillosis. Disease in all host species was often refractory to aggressive antifungal therapeutic regimens. Four other human isolates previously reported as *A. viridinutans* were identified as *A. felis* on comparative sequence analysis of the partial β-tubulin and/or calmodulin genes. *A. felis* is a heterothallic mold with a fully functioning reproductive cycle, as confirmed by mating-type analysis, induction of teleomorphs within 7 to 10 days *in vitro* and ascospore germination. Phenotypic analyses show that *A. felis* can be distinguished from the related species *A. viridinutans* by its ability to grow at 45 °C and from *A. fumigatus* by its inability to grow at 50 °C. Itraconazole and voriconazole cross-resistance was common *in vitro.*

The pathogenesis of URTA, in particular, the relationship between the infecting isolate and outcome, is poorly understood. Computed tomography was used to investigate the route of fungal infection and extension in 16 cases (SNA *n*=7, SOA *n*=9) where the infecting isolate had been identified by molecular testing. All cases had nasal cavity involvement except one cat with SNA that had unilateral frontal sinus changes. A strong association between the infecting species and anatomic form was identified. *A. fumigatus* infections remained within the sino-nasal cavity. Cryptic species infections were associated with orbital and paranasal soft-tissue involvement and with orbital lysis. These species were further associated with a mass in the nasal cavity, paranasal sinuses or nasopharynx. Orbital masses showed heterogeneous contrast enhancement, with central coalescing hypoattenuating foci and peripheral rim enhancement. Severe, cavitated turbinate lysis, typical of canine SNA, was present only in cats with SNA. These findings support that the nasal cavity is the portal of entry for fungal spores in feline URTA and that the route of extension to involve the orbit is via direct naso-orbital

communication from bone lysis. Additionally, a pathogenic role for *A. wyomingensis* and a sinolith in a cat with *A. udagawae* infection are reported for the first time.

Detection of the fungal antigen galactomannan in serum has poor sensitivity for diagnosis of URTA in cats. The diagnostic sensitivity of detection of *Aspergillus*-specific antibodies has not been determined. The aims of this study were (i) to determine if a commercial aspergillin derived from mycelia of *A. fumigatus, A. niger* and *A. flavus* can be used to detect serum antibodies against cryptic *Aspergillus* species and (ii) to assess the diagnostic value of detection of *Aspergillus*-specific antibodies using an agar-gel double immunodiffusion (AGID) assay and an indirect IgG ELISA. Sera from 21 cats with URTA (Group 1), 25 cats with other upper respiratory tract (URT) diseases (Group 2) and 84 control cats (Group 3) were tested.

Isolates from cats with URTA comprised *A. fumigatus* (5), *A. flavus* (1) and four cryptic species; *A. felis* (12), *A. thermomutatus* (1), *A. lentulus* (1) and *A. udagawae* (1). There were significantly more brachycephalic breed cats in Group 1 (52%) compared with Group 2 (16%, P=0.0125*)* or Group 3 (3%, P<0.001). Antibodies of cats infected with cryptic fungal species cross-reacted with the aspergillin. The AGID was positive in 43% of Group 1 cats and negative in all other cats. The IgG ELISA was positive in 90% of Group 1 cats and negative in 96% and 100% of Group 2 and 3 cats respectively, using a cut-off value of 9 Elisa Units/mL. Brachycephalic purebred cats are significantly more likely to develop URTA than other breeds of cats. Detection of *Aspergillus*-specific antibodies by IgG ELISA has high sensitivity and specificity for diagnosis of feline URTA.

Through this program of research understanding of epidemiological, clinical and aetiological aspects of feline aspergillosis have been more fully elucidated. Key findings include discovery of a novel aetiological agent, *A. felis* which causes upper respiratory (feline), invasive pulmonary (human) and disseminated invasive (canine) aspergillosis in both immunocompetent (feline) and immunocompromised (human, canine) host species

and is present at least in Australia and Europe. In cats, SOA is the most common anatomic form of disease and *A. fumigatus* infections remain confined to the sinonasal cavity, while SOA is associated with *A. felis* and other cryptic species infections. Cats with URTA are systemically immunocompetent and specific anti-*Aspergillus* IgG antibodies are readily detectable in the serum of infected cats. This research represents an important step in our understanding of feline aspergillosis.

Chapter 1. Introduction

This research journey began in 2006 when I was referred three unusual cases of upper respiratory tract disease in young domestic cats presenting to the Valentine Charlton Cat Centre. Subsequent investigations revealed that all three cases had aspergillosis affecting the nasal cavity and paranasal sinuses, with additional orbital involvement in two cases. At that time very little was known about this disease in cats since only 12 cases had been described worldwide (Goodall et al., 1984; Hamilton et al., 2000; Peiffer et al., 1980; Tomsa et al., 2003; Whitney et al., 2005; Wilkinson et al., 1982). Fungal cultures had been performed in only four cases, of which only one had phenotypic identification to species level indicating infection with *Aspergillus niger* (Whitney et al., 2005). Coincidentally in 2006, colleagues at the Centre for Infectious Diseases at Westmead Hospital had requested submission of tissue specimens from humans and companion animals with mycoses to evaluate the diagnostic utility of a panfungal PCR assay that they had recently developed to amplify the internal transcribed spacer 1 (ITS1) region of the ribosomal DNA gene cluster (Lau et al., 2007). The molecular identification of biopsy tissues from one of my three feline cases was *Neosartorya pseudofischeri*. My interest was immediately piqued, and the ensuing program of research stemmed from these three sentinel cases.

The overall objective of my research was to characterise feline upper respiratory tract aspergillosis. Specific aims were to describe clinicopathological findings, identify aetiological agents, and evaluate diagnostic modalities and treatment outcomes. In order to achieve this, case materials including medical histories, computed tomographic images, fungal cultures and serological samples were recruited from affected cats Australia-wide initially (Chapter three), and subsequently from Europe and the USA (Chapters four and five). In Chapter three, the clinicopathological findings and treatment outcomes of 23 cats with aspergillosis from around Australia are described. The findings from this study set the stage for further research to confirm discovery of a novel species, *Aspergillus felis* (Chapter four). The specific aims of this part of the research

program were to identify isolates from clinically confirmed cases of feline aspergillosis using phenotypic, physiologic and phylogenetic analyses. Following on from the preliminary molecular identification of isolates described in Chapter three using comparative multilocus sequencing of the ITS1 region, I sequenced three partial genes from six *Aspergillus* isolates from cats with sino-orbital aspergillosis – betatubulin (*BenA*), calmodulin (*CalM*) and actin. Preliminary phylogenetic analyses of these results revealed the tantalizing possibility that most cases of feline sino-orbital aspergillosis were caused by a single novel species of *Aspergillus* closely related to *Aspergillus viridinutans*, a member of *Aspergillus* section *Fumigati.* After reading taxonomical studies published by Professor Robert Samson, a world-renowned authority on *Aspergillus* (Samson et al., 2007a), I contacted him and shared my preliminary findings, which sparked mutual excitement. I was fortunate to then spend six weeks at the CBS-KNAW Fungal Biodiversity Centre in Utrecht performing morphological and molecular studies of isolates with Professor Robert Samson, Dr Jos Houbraken and Tineke van Doorn, funded by an Endeavour Research Fellowship from the Australian Government. While in Europe I formed two further fruitful collaborations with Professor Malcolm Richardson from the Mycology Reference Centre in Manchester and Dr Maria Dolores Pinheiro. Between publication of the manuscript in Chapter three and that in Chapter four, a gripping nomenclature debate was playing out around the world concerning fungal taxonomy, which is addressed in the literature review in Chapter two.
Upon my return to Australia I collaborated with Dr Sarah Kidd, Director of the National Mycology Reference Centre (Adelaide), who performed antifungal susceptibility testing on the isolates described in Chapter four, to complete the phenotypic analyses.

The final part of the research program, outlined in Chapters five and six, turned back to the host in which *Aspergillus felis* was first discovered – the domestic cat. This was inspired by the finding that very few cases of sino-orbital aspergillosis have successful treatment outcomes and that most cats succumb to neurological involvement (Barrs et al., 2012b; McLellan et al., 2006; Smith and Hoffman, 2010). An understanding of disease pathogenesis is necessary to develop effective treatments. The specific aims of

the research described in Chapter five were to evaluate computed tomographic (CT) findings in cats with aspergillosis to determine whether all cats with orbital involvement have concurrent involvement of the sino-nasal cavity. A positive finding would support the putative route of infection via inhalation of spores and subsequent invasion of the orbit. Other aims were to determine if there was any association between CT features and the form of aspergillosis and/or infecting species. Around this time I concurrently supervised a Master's project (Whitney et al., 2013a) (Appendix two). Dr Joanna Whitney evaluated an immunoenzymatic sandwich ELISA to detect galactomannan (GM), a fungal cell wall antigen, in serum (Platelia™ *Aspergillus* EIA, Bio-Rad, Marnes-la-Coquette, France), for diagnosis of feline upper respiratory aspergillosis. She found that GM quantification by ELISA was not a reliable test for the early, non-invasive diagnosis of feline URT aspergillosis (sensitivity 23%, specificity 78%). Following on from this, I embarked on the final stage of my research program to evaluate the diagnostic utility of specific anti-*Aspergillus* antibody detection in serum, using agar gel immunodiffusion and Immunoglobulin G ELISA, for diagnosis of aspergillosis. The results of this research are outlined in Chapter six. Around this time I was invited to write a review on feline aspergillosis, and the discussion of the results of my research findings in Chapters three, four are integrated with other published literature in this review Chapter seven. Chapter seven also includes further discussion of the research findings of Chapters five and six and future directions for aspergillosis research.

Chapter 2. Literature Review

2.1 Respiratory Aspergillosis: A One Medicine Approach

Aspergillosis is a mycosis caused by infection with fungi from the Genus *Aspergillus* that affects a diverse range of animal species. It is diagnosed increasingly in humans due to an increased prevalence of immunosuppression from cancer, chemotherapy, organ transplantation and autoimmune disease (Romani, 2011). Further, global warming is predicted to increase the prevalence of fungal infections in mammals by increasing the geographic range of pathogenic fungi (Garcia-Solache and Casadevall, 2010). *Aspergillus* spp. are amongst the most common moulds on earth. These ubiquitous, filamentous, saprophytic, ascomycetes are distributed primarily in soil and decaying vegetation and have an important role in recycling environmental carbon and nitrogen (Latge, 1999). The genus is named after “aspergillum”, a brush or implement with a perforated head similar to a shower-head used by Roman Catholic priests for sprinkling holy water (Figure 1) (Bennett, 2009).

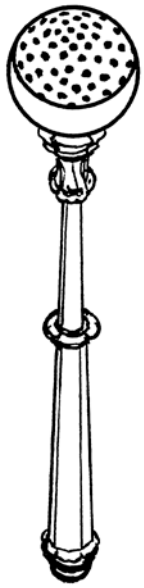


Figure 1: Line-drawing of an aspergillum, used by Catholic priests to sprinkle holy water. *Aspergillus* spp. reproduce asexually by producing spores or conidia on a morphological structure that resembles an aspergillum.

The respiratory tract is the most common site of disease reflecting the primary route of infection – inhalation. Infection involves the upper or lower respiratory tracts, is invasive or non-invasive as defined by presence or absence of tissue invasion by fungal hyphae and occurs in both immunocompetent and immunocompromised hosts. Invasive pulmonary aspergillosis (IPA) is the most common form of invasive aspergillosis (IA) in humans while upper respiratory tract (URT) aspergillosis, also known as fungal rhinosinusitis (FRS), is the most common form in cats and dogs. URT aspergillosis can be further subdivided into sinonasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA). In addition to feline URT aspergillosis, comparative aspects of respiratory aspergillosis in dogs and humans are reviewed.

2.2 Nomenclature and taxonomy

2.2.1 The Aspergillus fumigatus complex

The genus *Aspergillus* and the closely related genus *Penicillium* belong to the family *Aspergillaceae* (order *Eurotiales*, class *Eurotiomycetes*, phylum *Ascomata*). Several hundred species have been ascribed to the Genus *Aspergillus,* which is currently subdivided into 4 subgenera (*Aspergillus, Circumdati, Fumigati, Nidulantes)* and 19 sections (Houbraken et al., 2014) (Table 1.1).

Table 2.1

Subgenera and Sections in genus *Aspergillus (Houbraken et al., 2014)*

Subgenus	***Section* (teleomorph)**
Aspergillus	*Aspergillus* (Eurotium) *Restricti* (Eurotium)
Circumdati	*Candidi* *Circumdati* (Neopetromyces) *Flavi* (Petromyces) *Flavipedes* (Fennellia) *Nigri* *Terrei*
Fumigati	*Cervini* *Clavati* (Neocarpenteles, Dichotomomyces) *Fumigati* (Neosartorya)
Nidulantes	*Aeni* (Emericella) *Bispori* *Cremei* (Chaetosartorya) *Nidulantes* (Emericella) *Ochracerosei* *Silvati* *Sparsi* *Usti* (Emericella)

The most common isolates to cause respiratory aspergillosis in dogs, cats and humans are from the subgenus *Fumigati* section *Fumigati,* also known as the *Aspergillus fumigatus* complex (Balajee et al., 2007; Balajee et al., 2006; Barrs et al., 2012a; Peeters et al., 2008; Pomrantz and Johnson, 2007, 2010).

The *A. fumigatus* complex contains asexual members (anamorphs, mitotic phase), many of which also have sexual forms (teleomorphs, meiotic phase). The anamorph is typically mould-like and bears mitotic spores (conidia) while the teleomorph is characterised by the production of meiotic spores (ascospores) that develop within sacs (asci) that develop inside enclosed globose fruiting bodies known as cleistothecia (Samson et al., 2007a) (**Figure 2**).

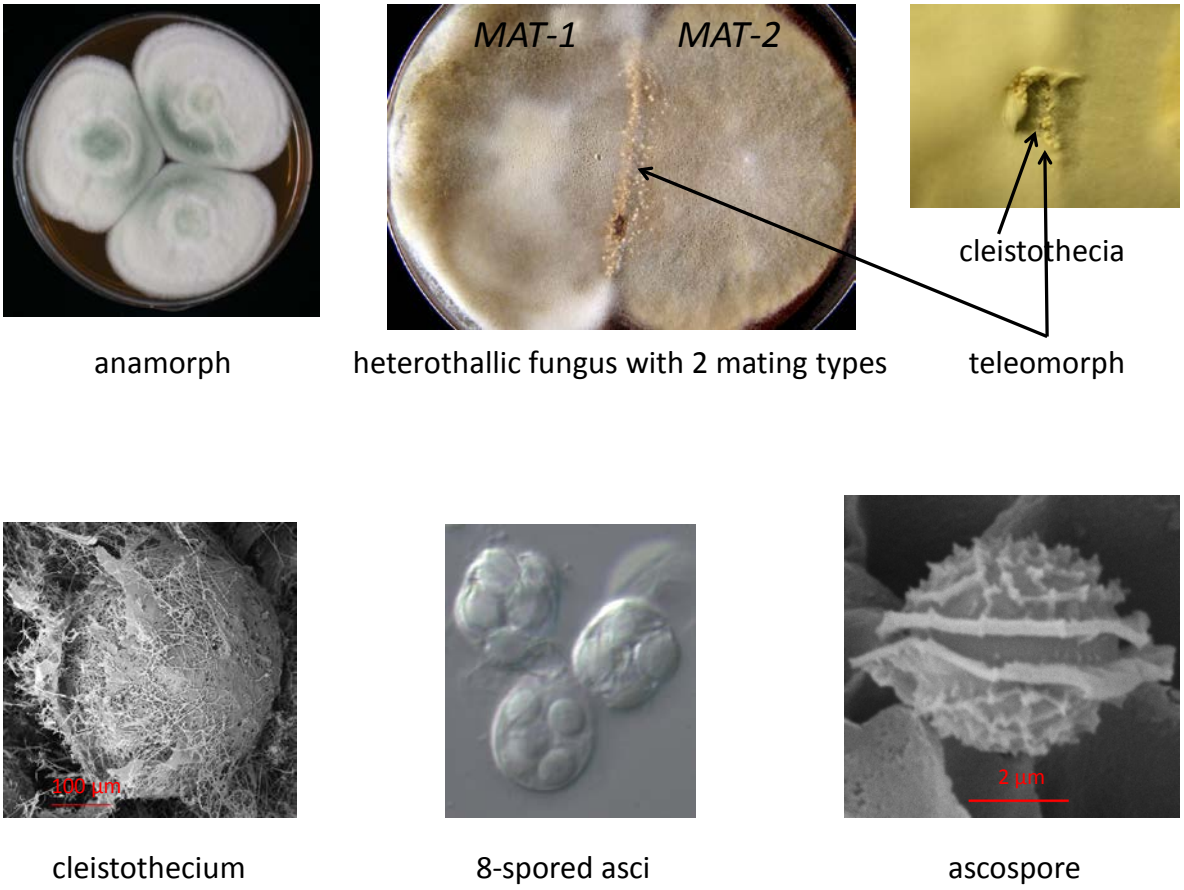


Figure 2. Holomorph of an *A. fumigatus complex* species, *A. felis sp. nov.* comprising anamorph and teleomorph phases. For heterothallic fungi the teleomorph forms at the junction of two colonies of complementary mating type.

Heterothallic fungi (e.g. *A. fumigatus*) require a partner of complementary mating type for successful sexual reproduction. The genes that determine mating-type (*MAT*) are located at a single locus, the *MAT-1* locus, which contains either a *MAT-1-1 "*idiomorph" or a *MAT-1-2* idiomorph (also known as *MAT-1* and *MAT-2* respectively) (Paoletti et al., 2005). *MAT-1* encodes a MAT protein with an alpha protein and *MAT-2* encodes a MAT protein with a high-mobility group (HMG) (Rydholm et al., 2007). The MAT proteins act as transcription factors to enable sexual reproduction. Homothallic species are self-fertilising and contain both the alpha and HMG-domain *MAT* genes within the same genome, either fused together or located in close proximity in the *MAT-1* locus, or within two distinct loci, as is the case for *Neosartorya fischeri* (Rydholm et al., 2007).

Controversy has surrounded the fungal taxonomy of the *Aspergillus fumigatus* complex because of the system of dual nomenclature used to describe anamorphic and teleomorphic phases of the same fungus. Traditionally the anamorphic phase is assigned to the genus *Aspergillus* while the teleomorph of the same organism is assigned to the genus *Neosartorya*. The entire organism, comprising the anamorph and teleomorph is referred to as the holomorph. Under classical nomenclature rules the teleomorphic name has received taxonomic precedence, such that species with known sexual stages were referred to by their teleomorph names (Pitt and Samson, 2007). While this system of dual nomenclature provided a practical solution for distinguishing organisms that produce ascospores, confusion arose for organisms such as *A. fumigatus* where the teleomorph (*Neosartorya fumigata*) was only recently discovered and the taxon continued to be referred to by its anamorph name (O'Gorman et al., 2009). In sweeping reforms to the *International Code of Nomenclature for algae, fungi and plants* (previously known as the *International Code of Botanical Nomenclature (ICBN) a* "one-fungus, one-name" principle was adopted in 2011 (Miller et al., 2011). The system of dual nomenclature was deleted, giving anamorph names the same priority as teleomorph names, and allowing anamorph names to be used as holomorph names. Subsequent to these reforms teleomorph-based genera such as *Neosartorya* are synonymised with *Aspergillus (Houbraken et al., 2014)*

2.2.2 Fungal species identification – a polyphasic approach

It is now well established that members of the *Aspergillus fumigatus* complex cannot be reliably identified solely on the basis of phenotypic features (morphological or physiological). Some, termed *A. fumigatus*-mimetic or "cryptic" species, have very similar anamorph colony morphology to *A. fumigatus.* For species delimitation, that is, determination of the boundaries and numbers of species from empirical data, and for identification of new species a polyphasic approach incorporating morphological, physiological, ecological and phylogenetic methods is considered the gold standard by mycologists since it is superior to any of these methods used in isolation (**Figure 3)** (Samson and Varga, 2009; Samson et al., 2007b).

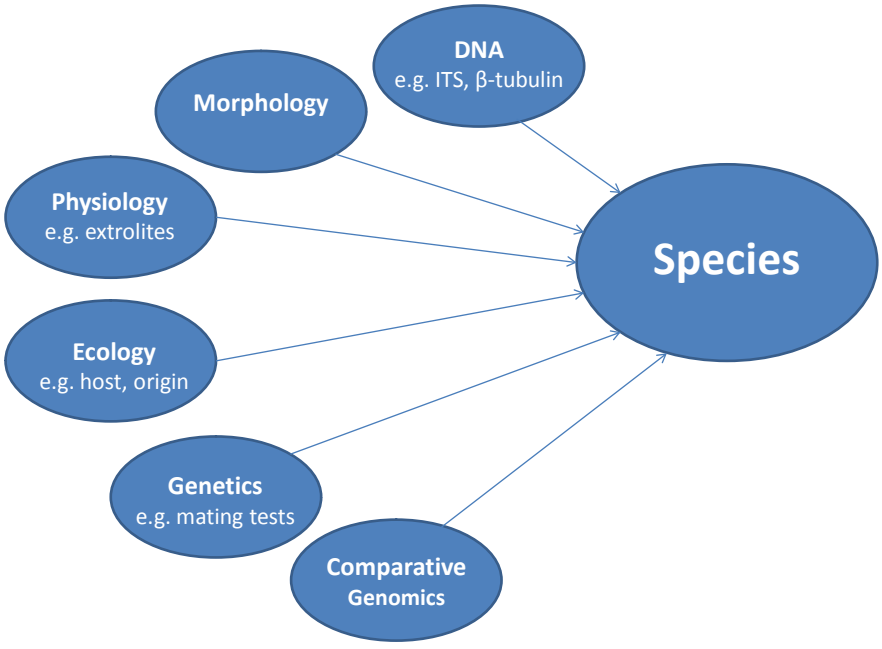


Figure 3. The use of combined data sets in polyphasic taxonomic classification of an *Aspergillus* taxon, after Samson and Varga (2009).

The polyphasic taxonomical approach for fungal species delimitation also encompasses three species concepts widely used in mycology (Rokas et al., 2007):

(i) Morphological species concept: species recognition based on morphological characters.

(ii) Biological species concept: species recognition based on establishment of reproductive isolation (applicable where teleomorphs are identified).

(iii) Phylogenetic species concept: species recognition based on shared ancestry.

Morphological and physiological methods for characterisation of *Aspergillus* species include macro- and micro-morphology, growth rate at different temperatures and water activities, growth on creatine-sucrose agar, conidial colour and secondary metabolite profiles. Ecological methods include geographic origin and host species while the most widely utilised phylogenetic method is multi-locus sequence typing (MLST) of loci such as the internal transcribed spacer (ITS) regions, β-tubulin, calmodulin, actin and other intron-rich protein coding genes (Balajee et al., 2007; Samson et al., 2007a; Samson et al., 2007b). Recommendations on the best-practice approach for describing new *Aspergillus* species including current requirements of the *International Code of Nomenclature for algae, fungi and plants* are listed in **Box 1** (Knapp et al., 2011; Samson and Varga, 2009; Samson et al., 2007b).

Box 1. Recommendations of an international panel of mycologists on best-practice approach to describing new *Aspergillus* species and updated requirements from the *International Code of Nomenclature for algae, fungi and plants*, after Samson et al 2007, Knapp et al 2011 and Miller et al 2011 (Knapp et al., 2011; Miller et al., 2011; Samson et al., 2007b)

- A polyphasic approach including molecular, morphological, physiological and ecological data should be used.
- New species should be compared to type strains of presumed relatives.
- Proposed new species should show evidence for evolutionary divergence from other taxa, particularly unique DNA characters at multiple independent loci (e.g. ITS, B-tubulin, calmodulin, actin, RNA polymerase), in addition to any distinctive extrolites and morphological characters.
- The description required for valid publication of the name of a new taxon may be in either English or Latin and should indicate differences from related taxa.
- Malt Extract Agar and Czapek's Agar, with referenced formulas, should be used for the description.
- Type cultures of new species should be deposited in at least two international recognized culture collections.
- If type cultures are not made available for the scientific community within 6 months of publication of the new species, the species will be considered invalid.
- A requirement for valid publication of a new species is a unique identifier issued through registration with a recognized repository, e.g. Mycobank www.mycobank.org/.
- Sequences must be deposited in recognized genetic databases.
- From 2012 the one fungus, one name principle will be adopted, where only a single name applies to each fungal species.

2.2.2.1 Macro- and micro- morphology

Phenotypic features routinely included in species descriptions for identification of *A. fumigatus* complex include colony patterns and the morphology of the conidiogenous structures, conidia, asci and ascospores. Colony patterns include colony diameter after 7-days growth of 3-point inoculations on Czapek agar, Czapek yeast autolysate agar (CYA), oatmeal agar (OA) and malt extract agar (MEA) at 25^{o}C and on CYA at 37^{o}C as well as colony colour, colony texture and reverse colony colour (Samson et al., 2007a).

Approximately a third of all described *Aspergillus* species have a known sexual phase (teleomorph) and less than 10 of these are heterothallic (Geiser, 2009). Heterothallic fungi within the *A. fumigatus* complex exist most commonly in their anamorphic form in nutritionally rich environments such as in nature and in the host. When conditions are unfavourable in nature for vegetative growth, sexual reproduction may be initiated (Pal et al., 2007). Cleistothecia of *Neosartorya* species in the *Aspergillus fumigatus* complex are characterised by walls consisting of flattened hyphae (**Figure 2**) (Geiser, 2009). Asci almost always contain eight ascospores, which are lens-shaped and often have two or more equatorial rings (**Figure 2**). Sclerotia, rounded masses of mycelium with an outer melanized rind, resemble cleistothecia grossly, but do not contain ascospores. Formation of sclerotia by some fungi is thought to facilitate survival during adverse growth conditions (Bennett, 2010).

Differences in ornamentation of conidia can assist in species identification (Balajee et al., 2007; Geiser, 2009). In a study of phylogenetic relationships among *A. fumigatus* and related species using comparative sequence analyses of partial regions of the β-tubulin, calmodulin and Rodlet A genes, five clades were identified (Yaguchi et al., 2007). Clades were correlated with conidial morphology using scanning electron microscope studies. Differences in conidial ornamentation were identified: in general isolates in clades I and V had lobate-reticulate ornamentation, isolates in clades II -IV

had microtuberculate ornamentation while the conidial ornamentation of *A. viridinutans* was intermediate between the other two types (Balajee et al., 2007; Yaguchi et al., 2007). The ornamentation of ascospores and their equatorial crests, of the teleomorphic species within the *A. fumigatus* complex, is highly but not exclusively species-specific since some species have similar ascospores (Samson et al., 2007a). Since the widespread implementation of phylogenetics less emphasis is now being placed on ascospore ornamentation in systematics (Geiser et al., 2007). Also, induction of teleomorphs in vitro is often difficult or impractical – some isolates cannot be induced to produce ascospores because of repeated subculturing on rich medium, induction of teleomorphs of heterothallic fungi requires possession of isolates of complementary mating type, and some species require prolonged incubation times of up to six months to induce ascospore formation, e.g. *A. fumigatus* (Balajee et al., 2005a; O'Gorman et al., 2009).

2.2.2.2 Mating tests

For heterothallic fungi the correlation between biological species based on mating tests and phylogenetic species based on multilocus sequence typing is very strong (Geiser et al., 2007).

2.2.2.3 Fungal growth temperature regimes

Differences in minimum and maximum growth temperatures using standardised media, usually MEA and CYA, are a useful phenotypic feature not only for species delimitation, but for identification of species in clinical settings. *A. fumigatus* is particularly thermotolerant being able to grow at temperatures over 50^{o}C. Ability to grow at 10^{o}C, 42^{o}C, 45^{o}C and 50^{o}C for a number of *A. fumigatus* complex members is depicted in Table 2.1 (Balajee et al., 2005b; Hong et al., 2005; Samson et al., 2007a; Sugui et al., 2010; Yaguchi et al., 2007).

Table 2. 1 Growth of *A. fumigatus* complex members at different temperatures

	Growth			
Species	**10**	**42**	**45**	**>50**
A. fumigatus	-	+	+	+
A. viridinutans	+	+	-	-
A. lentulus	+	+	+	-
N. udagawae	+	+	-	-

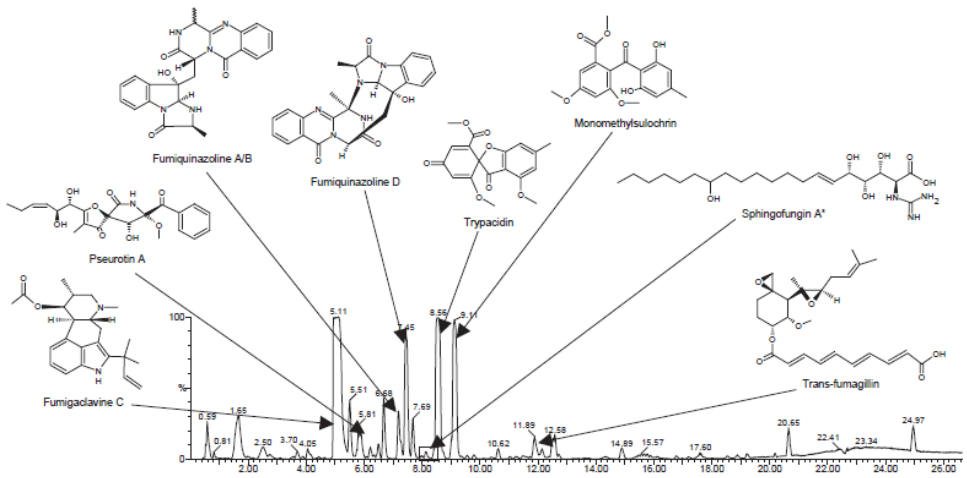
2.2.2.4 Metabolomics - Extrolite profiles

The metabolome of a fungus refers to its complete set of small-molecule metabolites. A small fraction of these molecules is utilised currently for the classification and identification of fungi (chemotaxonomy) based on profiles of outwards-directed metabolites (extrolites) as measured by chromatography and mass spectrometry (Frisvad et al., 2007; Hong et al., 2008). The secondary metabolites produced by *Aspergillus* species have high species-specificity - each species typically produces a unique combination of extrolites or a unique extrolite (Hong et al., 2008). Typical secondary metabolites produced by all *Aspergillus* species include polyketides, non-ribosomal peptides, terpenoids and compounds of mixed biosynthetic origin (Frisvad et al., 2009; Samson and Varga, 2009). One limitation of chemotaxonomy is that while all strains of the same species produce the same profile of secondary metabolites, one or more secondary metabolites may not be expressed phenotypically in any given strain (Frisvad et al., 2009).

Chemotaxonomic analyses require standardised growth conditions including culture media, growth temperature and analysis during sporulation. Optimal culture media to achieve the highest concentration and number of secondary metabolites in filamentous

fungi are those containing high amounts of sucrose and glucose, yeast or malt extract and added minerals and trace metals, e.g. dichloran-Rose Bengal-yeast extract-sucrose (DRYES), yeast extract-sucrose (YES) agar, malt extract agar (MEA) and potato-dextrose agar (PDA) (Hong et al., 2008). For comparative chemotaxonomy metabolite extraction from isolates should be performed at the same time using isolates grown together at the same temperature using the same batch of media (Hong et al., 2008). It has been recommended that for species descriptions, a panel of 4 to 8 extrolite be used (Samson et al., 2007b). In their revision of the taxonomy of *Aspergillus* section *Fumigati* Samson et al (Samson et al., 2007a) performed extrolite profiling using the high performance liquid chromatography (HPLC)-diode array detection method (Smedsgaard, 1997). Extrolites were analysed from cultures grown on YES, oatmeal agar (OA) and Czapek yeast autolysate agar (CYA) using agar plugs. In a recent update of the extrolite profile of *A. fumigatus* 226 extrolites from 24 biosynthetic families were detected (Frisvad et al., 2009). The major extrolites from *A. fumigatus* are depicted in **Figure 4**.

Figure 4. Extrolite profile of *A. fumigatus* depicting major and typical extrolites for this species, as described by Frisvad et al 2009. Reproduced with permission of Informa Healthcare (Frisvad et al., 2009).



2.2.2.5 Phylogenetic methods

Molecular characters provide the greatest number of variables for fungal taxonomy and can be generated using readily available technology. Phylogenetic methods that have been used for speciation of *Aspergillus* include RFLPs (restriction fragment length polymorphisms), AFLP (amplified fragment length polymorphisms), MLEE (multilocus enzyme electrophoresis), RAPD-PCR (random amplification of polymorphic DNA), ribosomal RNA sequences and protein-coding sequences (Geiser et al., 2007).

Multilocus comparative DNA gene sequence analysis of a combination of genes or partial gene regions including internal transcribed space (ITS), calmodulin, β-tubulin, actin and Rodlet A is the most common phylogenetic method applied for species delimitation and identification within the *A. fumigatus* complex (Samson et al., 2007a). The genome of all fungi contains multiple copies of the ribosomal DNA (rDNA) gene complex, consisting of highly variable regions, the ITS regions, which are flanked by highly conserved gene sequences that are suitable targets for primers. Sequence heterogeneity within the ITS regions is useful for the separation of both genera and species, and appropriately exploited as a “panfungal” PCR for identification of fungi in clinical specimens (Iwen et al., 2002; Lau et al., 2007). The rDNA gene complex includes three genes - the 18s rDNA gene, also known as the small-subunit (SSU) rDNA gene, which is about 1800 base pairs (bp) long, the 5.8S gene (159 bp) and the 28S rDNA, also known as the large-subunit (LSU) rDNA gene (3396 bp) (**Figure 5)** (Chen et al., 2002). Comparative sequence analyses of the ITS regions, specifically ITS1-5.8S-ITS2, is recommended as an appropriate locus to first identify *Aspergillus* isolates to the level of subgenus/complex (Balajee et al., 2007). However since some closely related species show little or no variation in ITS sequences, solid phylogenetic species recognition requires concurrent analyses of one or more of the aforementioned gene regions (Samson et al., 2007b). Samson et al (Samson et al., 2007a) found that

sequencing the calmodulin and β-tubulin genes achieved good species delimitation and recognition for *A. fumigatus* complex members.

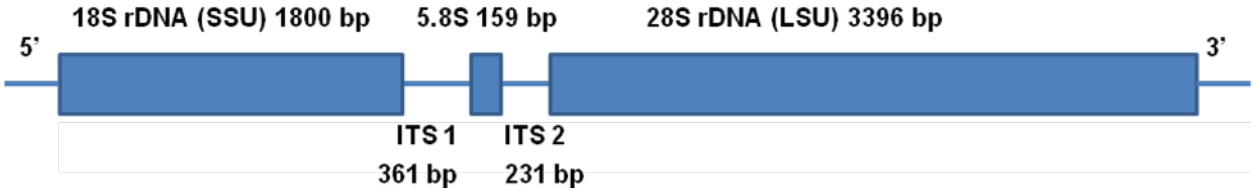


Figure 5. Representation of the ribosomal DNA (rDNA) gene complex in fungi denoting gene order of small subunit (SSU) and large subunit (LSU) and position of internal transcribed spacer (ITS) regions, after Chen et al., 2002 (Chen et al., 2002)

2.3 Aetiological agents of aspergillosis in humans, dogs and cats

2.3.1 Humans

A. fumigatus, A. flavus, A. terreus and *A. niger* are the most common causes of IA in humans (Klich, 2006; Thompson and Patterson, 2011). Of these *A. fumigatus* is reported most commonly. However, more recently with the application of molecular identification techniques, other members of the *A. fumigatus* complex, known as *A. fumigatus*-mimetic species or 'cryptic' species previously mis-identified as *A. fumigatus,* have been increasingly recognised in patients with IA, including *A. lentulus*, *A. viridinutans*, *A. udagawae*, *N. pseudofischeri*, *A. fumisynnematus* and *N. hiratsukae* (Alcazar-Fuoli et al., 2008; Balajee et al., 2005b; Balajee et al., 2006; Katz et al., 2005; Sugui et al., 2010; Vinh et al., 2009a). Fungal rhinosinusitis (FRS) is most commonly caused by *A. fumigatus* or *A. flavus* although confirmation of identification of isolates using molecular techniques has not yet been reported (Chakrabarti et al., 2009; Hope et al., 2005a).

Mis-identification of *A. fumigatus* in human patients with IA has important implications for treatment and prognosis. In a review of 86 isolates from human patients with IA previously identified as *A. fumigatus*, 12 isolates were subsequently identified as *A. udagawae* based on beta-tubulin and rodlet A sequences (Sugui et al., 2010). In this subset of patients the median duration of illness was 7 times longer and disease was refractory to standard therapy. Similarly, a distinctive form of IA characterised by chronicity, propensity to spread across anatomical planes and reduced susceptibility to antifungal drugs was initially attributed to infection by *A. fumigatus*. The molecular identity was *A. viridinutans* (Vinh et al., 2009a). Also *A. fumigatus*-mimetic species have higher *in vitro* minimum inhibitory concentrations (MICs) for amphotericin-B and triazole antifungal drugs (e.g. voriconazole) than *A. fumigatus* (Alcazar-Fuoli et al., 2008).

2.3.2 Dogs

Phenotypic identification of a large number of isolates from dogs with SNA has implicated *A. fumigatus* as the aetiologic agent in most cases (Pomrantz and Johnson, 2007, 2010). There are also occasional reports of isolates from the subgenus *Circumdati (A. flavus* and *A. niger*) (Sharp, 1998) and from the subgenus *Nidulantes* (*A. nidulans*) (Sharp et al., 1991b) causing SNA in dogs. However, identification of isolates was phenotypic in all but one report where molecular identification was performed on a small number of isolates only (Peeters et al., 2008). In 14 dogs with clinically confirmed SNA, DNA of *Penicillium* or *Aspergillus* spp. was detected in nasal mucosal biopsies using a genus-specific ITS real-time quantitative PCR (qPCR) assay. Species-specific ITS qPCRs to detect DNA of *A. fumigatus, A. niger, A. terreus* or *A. flavus* developed by Haugland et al (Haugland et al., 2004) were positive for *A. fumigatus* in 7 dogs and negative in the other 7 dogs. It is possible that the negative isolates may have been *Penicillium* spp. or another *Aspergillus* spp. Alternatively there may have been insufficient *A. fumigatus* DNA present to be positive with the specific PCR. Fungal culture was positive in only 6 of the dogs diagnosed with aspergillosis, of which DNA of *A. fumigatus* was detected in 5. Also on examination of histological Grocott-stained sections, fungal hyphae were observed at the surface of the mucosa in biopsies from

only 6 of the 14 dogs. A likely reason for the negative culture and histologic results in these dogs was that a standardized site for biopsy of sinonasal mucosa was used for biopsy collection. Since the distribution of fungal plaques within the sinonasal cavity is uneven, fungal plaques were likely missed using this sampling approach. Furthermore, seven dogs had also received oral antifungal therapy (ketoconazole or itraconazole) for 8 to 15 days prior to biopsy collection, which could have contributed to the negative culture, histologic and PCR results in affected dogs. Clearly there is a need for molecular studies to determine the aetiological agents in canine sinonasal aspergillosis, specifically the frequency of involvement of individual species within the *Aspergillus fumigatus* complex and from subgenera other than *Fumigati*.

2.3.3 Cats

Little information exists about the aetiological agents of feline upper respiratory tract (URT) aspergillosis. Of 22 reported cases fungal culture was not attempted in four cases (Peiffer et al., 1980; Smith and Hoffman, 2010; Whitney et al., 2005) and was negative in six others (Goodall et al., 1984; Halenda and Reed, 1997; Tomsa et al., 2003; Whitney et al., 2005). Of the 12 cases in which fungal culture was positive, identification was based on phenotypic features alone in 11 cases and on both phenotypic features and molecular identification in one case only. *A. fumigatus* was identified in five cases (Barachetti et al., 2009; Giordano et al., 2010; McLellan et al., 2006; Quimby et al., 2010; Smith and Hoffman, 2010), an *Aspergillus* of unknown species was identified in three cases (Furrow and Groman, 2009; Hamilton et al., 2000; Wilkinson et al., 1982), *A. niger* was identified in two others (Furrow and Groman, 2009; Whitney et al., 2005) and *A. flavus* in one case (Malik et al., 2004). *A. udagawae* was identified as a cause of feline SOA in a single case report from Japan using comparative sequence analysis of the ITS and β-tubulin genes (Kano et al., 2008).

2.4. Aspergillosis - parallels between humans, dogs and cats with regard to epidemiology, pathogenesis and clinical disease.

2.4.1 Humans

In humans aspergillosis is generally classified by body system involvement, pathology and pathogenesis. Disease is subcategorized, according to the presence or absence of hyphal invasion into tissues, as invasive aspergillosis (IA) or non-invasive aspergillosis. IA is further classified according to the presence or absence of angioinvasion, i.e. fungal invasion of blood vessels (Hope et al., 2005a). Invasive aspergillosis (IA) in humans occurs predominantly in the sinopulmonary tract of immunocompromised individuals, after inhalation of *Aspergillus* spp. conidia (Segal, 2009). The lung is the most common site of disease, with invasive pulmonary aspergillosis (IPA) accounting for over 90% of IA cases (Hope et al., 2005a). The classification of sinopulmonary and disseminated forms of aspergillosis is summarised in **Table 2.2** (Chakrabarti et al., 2009; Hope et al., 2005a; Panda et al., 2004; Uri et al., 2003).

Table 2.2 Classification of disseminated and respiratory aspergillosis in humans.

Anatomic Location	Invasive/ Non-invasive	Immune status	Features of Disease
Disseminated	Invasive	Immunocompromised	Infection at ≥ 2 non-contiguous sites, haematogenous dissemination.
Lower Respiratory Tract	Invasive Pulmonary Aspergillosis (IPA) (Angioinvasive)	Immunocompromised - prolonged severe neutropenia	Vascular invasion by fungal elements
	IPA (Non-Angioinvasive)	Immunocompromised non-neutropenic: - HIV/AIDS - corticosteroids - hematopoietic stem cell transplant recipients - heritable immune defect	No evidence of vascular invasion. Pyogranulomatous inflammatory infiltrate.
	Chronic Invasive PA (Non-Angioinvasive): - chronic necrotizing PA - chronic cavitary PA - chronic fibrosing PA	Immunocompromised: - structural lung disease e.g., emphysema - corticosteroids - HIV/AIDS - diabetes, alcohol abuse	Hyphae mostly contained within cavity with only occasional direct tissue invasion (chronic necrotizing PA).
	Invasive bronchial aspergillosis	Immunocompromised	Hyphal invasion of large airway(s).
	Aspergilloma - non-invasive	Immunocompetent	Single cavity with fungal ball. No hyphal invasion of parenchyma.
Upper Respiratory Tract	Acute invasive fungal rhinosinusitis (FRS) (Angioinvasive)	Immunocompromised: - Neutropenia - Allogenic stem cell transplant recipients	Hyphal invasion of sinuses and contiguous structures e.g. orbit. Coagulative necrosis, sparse inflammatory infiltrate, angioinvasion.
	Chronic invasive FRS (Angioinvasive)	Immunocompromised: - diabetes - corticosteroids - HIV/AIDS	Hyphal invasion of sinuses and contiguous structures e.g. orbit. Infiltrative mass, mixed inflammatory response, angioinvasion.
	Granulomatous FRS - invasive	Immunocompetent: - Location dependent disease Sudan, Middle-East, Indian subcontinent	Hyphal invasion of sinuses and contiguous structures e.g. orbit. Highly cellular granulomatous inflammatory response, no angioinvasion
	Sinus aspergilloma (fungal ball) - non-invasive	Immunocompetent: - structural sinus disease	Fungal mass within sinus. Chronic non-granulomatous inflammatory response to fungal mass.
	Allergic FRS - non-invasive	Immunocompetent	Allergic/hypersensitivity response to the presence of fungi within the sinus.
	Chronic erosive non-invasive FRS	Immunocompetent	Similar to canine SNA. Marked inflammatory response and sinonasal bony lysis but no hyphae invasion of mucosa.

FRS is defined as invasive when there is hyphal invasion of the mucosal layer. Invasive forms of FRS include acute invasive, chronic invasive and granulomatous FRS while non-invasive forms include fungal ball or sinus aspergilloma, allergic FRS and chronic erosive non-invasive FRS (Day, 2009; Hope et al., 2005a) (Table 1.2). FRS in humans includes infections caused by *Aspergillus* and other filamentous fungi including *Mucor* spp. (hyalohyphomycoses) and the dematiaceous fungi (phaeohyphomycoses). Further discussion of granulomatous FRS and chronic erosive non-invasive FRS is warranted here given disease similarities of granulomatous FRS to SOA in cats and of chronic erosive non-invasive FRS to SNA aspergillosis in dogs:

2.4.1.1 Chronic granulomatous fungal rhinosinusitis (FRS)

Granulomatous FRS occurs in immunocompetent humans subjected to hot, dry environmental conditions, poor hygiene and low socioeconomic status (Chakrabarti et al., 2009; Siddiqui et al., 2004). This syndrome has mainly been reported in Asia and Africa (Pakistan, India, Saudi Arabia, Sudan, other African countries). In contrast to other forms of FRS in humans that are mostly caused by *A. fumigatus,* chronic granulomatous FRS is predominantly caused by *A. flavus,* which has a propensity to colonise the nasal and paranasal sinuses in hot, dry climates (Siddiqui et al., 2004). Disease is characterised by formation of a necrotic, friable or purulent mass within the sinuses followed by invasion of contiguous structures such as the orbit and/or brain, and by bony lysis. Clinical presentation includes nasal congestion or discharge, proptosis (exophthalmos), restriction of extraocular movements and decreased vision. Disease is painful in a third of cases and neurological deficits occur where there is CNS invasion (Pushker et al., 2011; Siddiqui et al., 2004). Fungal granulomas in affected patients are relatively avascular, consist of a highly cellular inflammatory infiltrate of giant cells, histiocytes, plasma cells and fungal hyphae and are necrotic or fibrotic. Angioinvasion does not occur, although blood vessels are thickened (Hope et al., 2005a).

2.4.1.2 Chronic erosive non-invasive fungal rhinosinusitis (FRS)

Chronic erosive non-invasive FRS of humans was first described by Rowe-Jones and Moore-Gillon in 1994. Disease is characterised by sinus expansion and bony erosion but not tissue invasion, resulting in chronic inflammation intermediate between sinus aspergilloma and chronic invasive FRS (Rowe-Jones and Moore-Gillon, 1994). However the classification of chronic erosive non-invasive FRS is not without controversy, with some investigators suggesting it is really a variant of allergic FRS (Chakrabarti et al., 2009). Despite this, chronic erosive non-invasive FRS of humans bears many similarities to canine SNA (Panda et al., 2004; Uri et al., 2003) such that dogs have been suggested as an appropriate model for study of this disease (Day, 2009; Peeters et al., 2005). Similarities between human and canine disease include chronicity, systemic immunocompetence, the non-invasive nature of the infection with fungal hyphae adjacent put not penetrating the sinonasal mucosa, the often extensive sinonasal bony lysis and to some degree the histological findings. In both species there is an intense mucosal inflammatory reaction to the presence of fungi, which in humans consists of neutrophils, lymphocytes and histiocytes, whereas the infiltrate in dogs is predominantly lymphoplasmacytic with admixed macrophages and fewer neutrophils (Day, 2009).

2.4.2 URT aspergillosis in dogs and cats

2.4.2.1 Pathogenesis

In contrast to humans, mycoses with an inhalational route of infection such as aspergillosis and cryptococcosis preferentially cause rhinosinusitis over pulmonary infection in cats and dogs. This may be associated with anatomical differences in nasal cavity and paranasal sinus conformation resulting in initial deposition of inhaled fungal spores within the sinonasal cavity in cats and dogs compared to the lower respiratory tract in humans. The ability of fungi to cause disease depends on a complex interplay between the pathogen (virulence factors) and the host (innate and adaptive immune responses).

2.4.2.1.1 Innate anti-fungal immunity

The first line of defense against fungal respiratory infection is the mucosal epithelium of the respiratory tract that traps and removes inhaled conidia via the mucociliary escalator and acts as an anatomic barrier to invasion (Segal, 2009). Phagocytosis of fungal elements, the next line of defense, by certain host cells (monocytes, macrophages, neutrophils and respiratory epithelial cells) results in direct pathogen killing in contrast to dendritic cells (DCs) that respond to phagocytosis by promoting the differentiation of naïve T cells into effector T helper (Th) cell subtypes (adaptive immunity) **(Figure 6)** (Romani, 2011).

After inhalation, *Aspergillus* conidia that escape mucociliary clearance are mostly phagocytosed by macrophages and DCs. Macrophages ingest and kill conidia preventing germination to hyphae, which are invasive. Hyphae resulting from germination of conidia that fail to be killed by macrophages, pierce through the cell and grow extracellularly. Neutrophils are the dominant host defense against hyphae, which are too large to be phagocytosed completely and must be killed by extracellular mechanisms. Activation of NAPDH oxidase in macrophages and neutrophils is important for conidial and hyphal killing respectively (Shoham and Levitz, 2005). Natural killer cells are also important in host-defense and are recruited to the site of infection by chemokines (Segal, 2009). Humoral factors involved in the host response to *Aspergillus* include activation of the complement cascade and deposition of complement components on the surface of conidia and hyphae. For example complement receptor 3 (CR3) recognizes C3b or C3d coated β-glucan-containing cell wall components (Romani, 2011).

Phagocytic host cells express **pattern recognition receptors (PRRs)** that recognise specific fungal epitopes known as **pathogen associated molecular patterns (PAMPs)** and damaged host cell components known as **damage associated molecular patterns (DAMPs)**. The major PAMPs of filamentous fungi are cell-wall components

including β-glucans, chitin and mannans while DAMPS include nucleic acids and alarmins.

The major PRRs of host cells include:
(i) **C-type lectin receptors** (CLRs) – CLRs are a family of transmembrane and soluble proteins that have at least one carbohydrate-recognition domains, including dectin-1 and dectin-2 and the complement-activating mannose-binding lectins, which are involved in antifungal immunity. Dectin-1 is the main PRR for β-glucan. Humans with genetic deficiencies of dectin-1 are highly susceptible to IFIs. Ligation of dectin-1 results in pro- and anti- inflammatory cytokine and chemokine production and can also stimulate NADPH oxidase activation (Romani, 2011; Segal, 2009). The NADPH oxidase in phagocytes results in the conversion of oxygen to superoxide anion and the generation of downstream reactive oxidant metabolites with antimicrobial activity (Segal, 2009).

(ii) **Toll-like receptors** (TLRs) – TLRs are a family of transmembrane proteins that recognise both PAMPs and DAMPs. TLRs signal the presence of pathogen or tissue damage to the host. TLRs are homologous to interleukin-1 receptor 1 and lead to the activation of transcriptional factor nuclear factor and mitogen-activated protein kinases. In general, activation of TLRs by fungi results in the expression of pro-inflammatory cytokines (Segal, 2009). TLR2, TLR4 and TLR9 are the major TLRs involved in recognition of fungal PAMPs. A polymorphism in TLR4 in humans is associated with increased susceptibility to IPA (Romani, 2011). The morphological phase of the fungus encountered by the host-cell has a dramatic effect on differential TLR activation and the resultant signaling pathways. When macrophages encounter *Aspergillus* conidia, TLR4-dependant pathways are stimulated, resulting in the production of pro-inflammatory cytokines such as TNF-α, IL-1α and IL-1β. By contrast when macrophages encounter *Aspergillus* hyphae, TLR2-dependant pathways are activated resulting in IL-10 production, an anti-inflammatory cytokine (Shoham and Levitz, 2005).

(iii) **Nucleotide Oligomerization Domain-like receptors** (NOD-like receptors or NLRs). NLRs are a family of cytoplasmic proteins that recognise PAMPS and endogenous ligands. They have a role in regulation of both inflammatory and apoptotic responses. The specific NLRs involved in host recognition of fungi are yet to be described.

(iv) **Galectin family proteins** – e.g. galectin 3 recognises the PAMP of β-mannisidoses in the fungal cell wall.

Host factors that predispose individuals to IA include disorders of innate immunity such as reduced mucociliary clearance (as occurs with cystic fibrosis), decreased numbers of phagocytic cells (i.e. neutropenia) and phagocytic dysfunction (e.g. chronic granulomatous disease, in which there is impaired production of oxidative intermediates). Long term administration of corticosteroids increases susceptibility to IA because of decreased NAPDH activation in phagocytes and also by suppression of production of IL-1α, CD14 and MyD88, which are protective against aspergillosis (Shoham and Levitz, 2005).

2.4.2.1.2 Adaptive anti-fungal immunity

Dendritic cells (DCs) modulate the antifungal host response. After exposure to *Aspergillus* conidia and hyphae DCs migrate to the spleen and draining lymph nodes inducing specific Th responses which are an essential determinant of host susceptibility or resistance to IA (Shoham and Levitz, 2005). In the immune-response to *Aspergillus* Th1 cytokine responses are generally protective while Th2 cytokine responses increase susceptibility to IA.

(i) **Th1 responses** – a dominant Th1 cell response correlates with protective antifungal immunity. Activation of Th1 cells is determined by the response of DCs to the combination of TLR and CLR signals provided by the fungi. Through production of the signature cytokine IFNδ and by aiding production of opsonizing antibodies, Th1 cells

enable optimal activation of phagocytes at sites of infection (Romani, 2011). In addition to IFNδ other proinflammatory signals characteristic of a Th1 protective response include granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-1, IL-6, and IL-12, IL-18 as well as the chemokines MIP-1, monocyte chemoattractant protein (MCP)-1, and MIP-2 (Shoham and Levitz, 2005). In experimental mouse models of IPA augmentation of Th1 responses by administration or depletion of specific cytokines enhances antifungal host defense (Cenci et al., 1998).

(ii) **Th2 responses** – IL-4 and IL-13 are the strongest proximal cytokines to promote differentiation of naïve T cells intoTh2 cells (Romani, 2011). Cytokines produced by Th2 cells include IL-4 and IL-5 **(Figure 6).** Th2 responses favor fungal invasion, fungus-associated allergic responses (e.g. allergic bronchopulmonary aspergillosis and disease relapse (Segal, 2009).

(iii) **Th17 response**s – The Th17 pathway has a regulatory function in the promotion of Th1 responses and suppression of Th2 responses. Th17 cells express mediators to recruit neutrophils and produce defensins at sites of infection **(Figure 6)**. Th17 responses are also implicated in the immunopathogenesis of chronic fungal infections possibly by failure to restrain inflammation after IL-17A dependent neutrophil recruitment (Romani, 2011).

(iv) **T regulatory (Treg) cell responses** – The decision of how to respond to infection is primarily determined by interactions between pathogens and cells of the innate immune system. However, Treg cells feed back into this dynamic equilibrium to regulate subsequent immune responses (Romani and Puccetti, 2006). Usually, Treg cells serve to restrain exuberant immune reactivity, which benefits the host by limiting tissue damage in many chronic infections. However, Treg cell responses can handicap the efficacy of protective immunity. Treg cells are a principal source of IL-10 in addition to cells infected or exposed to fungal PAMPs (Romani, 2011; Romani and Puccetti, 2006).

Thus Treg cell responses are responsible for a spectrum of outcomes ranging from protective tolerance to decreased cellular immunity.

Role of IL-10 in fungal infections

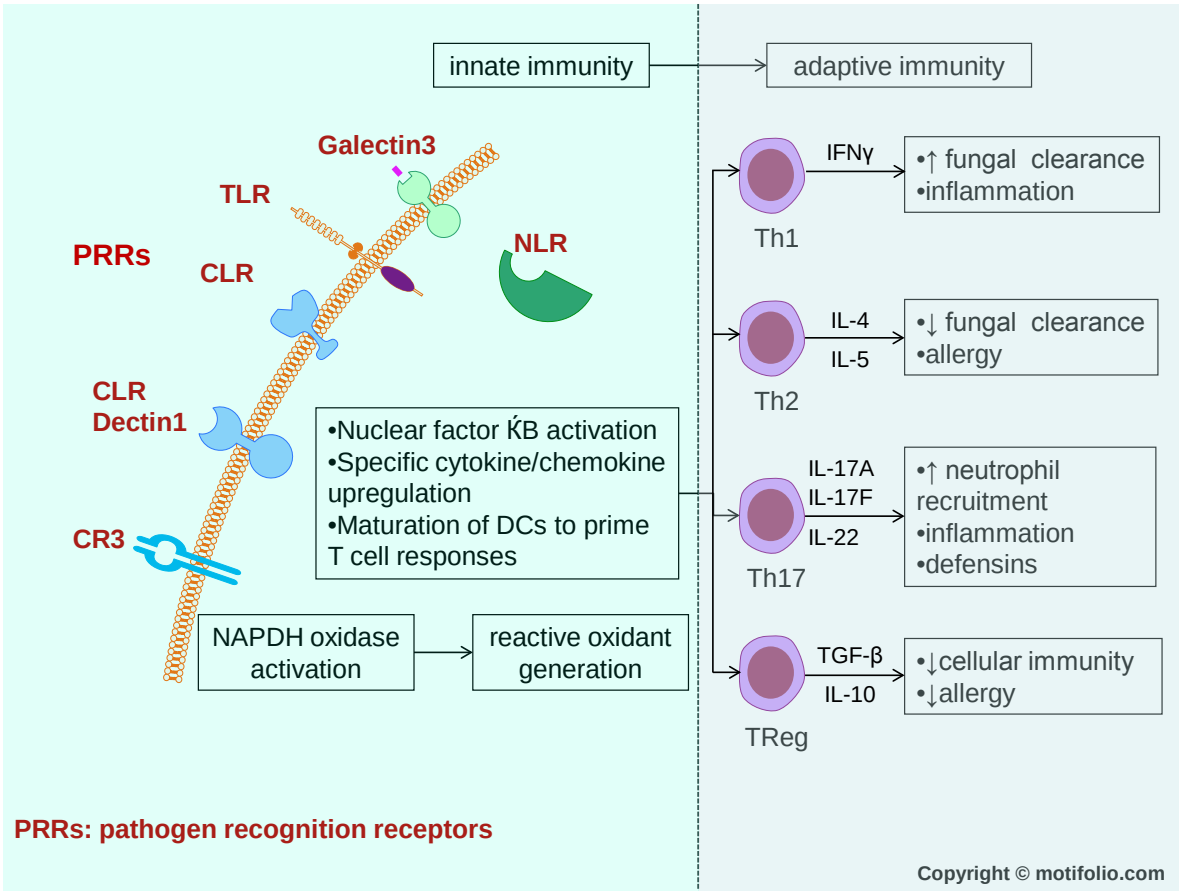
IL-10 is produced by T cells from healthy humans in response to fungal antigen preparations, a finding that establishes IL-10 as a signature cytokine of the T-cell repertoire in response to fungi (Romani and Puccetti, 2006). In patients with chronic IFIs there is an inverse relationship between IFNδ (signature Th1 cytokine) and IL-10. High levels of IL-10, present in neutropenic patients with IA negatively impact the production of IFNδ and have been linked to susceptibility to IFI (Hebart et al., 2002; Romani, 2011). Further, in one study where serial serum IL10 levels were measured, patients that had undetected or low levels of IL-10 or that had high levels initially that became undetectable had favorable outcomes to therapy in contrast to patients that had progressive disease in which IL-10 levels increased (Roilides et al., 2001).

However, given its prominent effect on resolving inflammation, IL-10 production can be a consequence rather than a cause of infection. In chronic fungal infections dominated by non-resolving inflammation IL-10 acts as a homeostatic host-driven feedback mechanism to limit pathogenic inflammatory responses. The inflammatory process in fungal infection is beneficial in containing the infection but an uncontrolled inflammatory response is detrimental and may eventually oppose disease eradication (Romani, 2011). Even in the presence of IL-10, the protective effect of a regulated Th1 response is largely preserved, although in some instances, eradication of the pathogen can be prevented.

2.4.2.1.3 Fungal virulence factors and evasion of the immune system

The fungal cell wall changes in structure depending on the morphological phase of the fungus. β-1-3-glucans are masked on hyphae and can evade detection by dectin-1, while coating of *A. fumigatus* conidia by melanin and hydrophobins is another mechanism that helps evasion of immune recognition (Romani, 2011).

Figure 6: Model of immune response to inhaled *Aspergillus* spp., after Segal et al 2009 and Romani 2011. Ligation of pathogen recognition receptors (PRRs) of host macrophages and respiratory epithelial cells by fungal pathogen associated molecular patterns (PAMPs) results in induction of chemokines/cytokines that recruit neutrophils and other inflammatory cells. Dendritic Cells (DCs) recognise PAMPs via PRRs and stimulate antigen-dependent responses in T helper (Th) and T regulatory (Treg) cells (Segal, 2009)



2.4.2.1.4 Immunology of canine SNA

Several studies have evaluated the immune response to SNA in dogs (Mercier et al., 2012; Peeters et al., 2006; Peeters et al., 2007; Vanherberghen et al., 2012). In the first study the mucosal immune response in SNA was compared to dogs without nasal cavity disease through the quantification of messenger RNA (mRNA) encoding cytokines and chemokines in nasal mucosal biopsies (Peeters et al., 2006). There was significantly more IL8, IL-10, IL-18, TNFα, and monocyte chemoattractant proteins (MCP) 1 – 4 mRNA expression in the nasal mucosa of dogs with SNA compared to dogs with normal nasal mucosa. Given that a dominant Th1 response is associated with production of IFNδ by Th1 cells and of the proinflammatory cytokines IL-12, IL-18 and TNFα, the upregulation of IL-18 and TNFα in dogs with SNA was considered compatible with this type of response (Peeters et al., 2006). A dominant Th1 response is likely important in preventing invasive disease. IL-10 is a potent immunosuppressive cytokine that exerts both beneficial and detrimental effects on host responses to fungi as discussed previously. Upregulation of IL-10 might be important in limiting the extent of local tissue destruction (Romani, 2004), but paradoxically could also be the reason why affected dogs are unable to clear infection spontaneously. The finding of upregulation of IL-10 in dogs with SNA was corroborated by a second study in which the immune response to SNA and to lymphoplasmacytic rhinitis (LPR) was compared to determine whether the pathogenesis of these diseases was distinctive (Peeters et al., 2007). Quantitative reverse transcriptase PCR (qRT-PCR) was carried out on RNA extracted from nasal biopsies from dogs with SNA, LPR and control dogs using primers to amplify messenger RNA (mRNA) encoding a panel of cytokines and chemokines. SNA was associated with significantly increased expression of mRNA encoding IL-6, IL-8, IL-10, IL-12p19, IL-12p35, IL-12p40, IL-18, IFN-δ, TNF-α, TGF-β, eotaxin-2 and all four MCPs relative to controls. LPR was associated with significantly increased expression of mRNA encoding IL-5, IL-8, IL-10, IL-12p19, IL-12p40, IL-18, TNF-α, TGF-β, MCP-2 and MCP-3 relative to controls. There was significantly more expression of mRNA encoding IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IL-18, IFN-δ, TNF-α, TGF-β and all MCPs, and

significantly less expression of IL-5 in dogs with SNA than in dogs with LPR. A key finding of this study was that the immunopathogenesis of SNA and LPR in dogs is different, in so far as dogs with LPR mounted a partial Th2 response, while the study gave further support that dogs with SNA mounted a Th1 response. This was an important finding as it clearly established that cases of LPR do not represent failure of diagnosis of SNA, as has been proposed previously (Peeters et al., 2007). Others have proposed LPR could represent a hypersensitivity response to colonization of the nasal cavity by fungal or bacterial pathogens in others (Windsor et al., 2006). The finding of upregulation of IL-5 in dogs with LPR supports this contention. The finding of marked upregulation of IFN- δ expression in dogs with SNA was important further evidence that this disease is characterised by a Th1 response. Furthermore, the upregulation of IL-10 and TGF-β in dogs with SNA was proposed as contributing to failure of clearance of infection (Peeters et al., 2007).

In order to explore the hypothesis that a dysfunction in innate immunity could be an aetiological factor in the development of SNA a further study quantified the expression of messenger RNA (mRNA) encoding two types of PRRs - Toll-like receptors (TLRs) 1–10 and NOD like receptors (NLRs) 1 and 2 in nasal mucosal biopsies from dogs with SNA or no evidence of nasal cavity disease using qRT-PCR normalized against multiple housekeeping genes (Mercier et al., 2012). The study demonstrated that TLRs 1-10 and both NLRs are expressed in the nasal mucosa of normal dogs. In dogs with SNA there was significantly higher expression of all PRRs except for TLR5 and NLR1 compared to normal dogs. The significance of these findings is unknown as little is known about the function of PRRs in nasal immunity in health and disease. This was the first study to demonstrate the expression of mRNA encoding all TLRs in canine tissue. The finding of TLR5 interest is of interest since it has also been shown to be down-regulated in humans and dogs with inflammatory bowel disease, associated in dogs with three different single nucleotide polymorphisms in the TLR5 gene (SNPs) (Kathrani et al., 2010).

In a more recent study, the functional phenotype of peripheral blood mononuclear cells (PBMCs) from normal dogs and dogs with SNA after challenge with heat-inactivated *A. fumigatus* conidia was investigated (Vanherberghen et al., 2013). Gene expression for specific Th1, Th2, Th17 and Treg cytokines and their related transcription factors was quantified. IL-4 and IFN-δ protein in culture supernatant was measured by ELISA. The study found that PBMC from dogs with SNA produced adequate mRNA encoding IFN-δ and IFN-δ protein. There was significantly greater expression of IL17A mRNA in the PBMC from affected v control dogs. In contrast to previous studies where IL-10 expression in nasal mucosal biopsies was increased, the amount of IL-10 mRNA in PBMC from dogs with SNA decreased after conidial challenge. The authors proposed that this could have been due to the absence of local inflammatory factors in PBMC cultures, or that IL-10 production may only occur in early stages of infection. It was proposed that an uncontrolled inflammatory response driven by IL-17A synthesis could counteract the host's efforts to clear fungal infection through Th-1 directed immunity. Another hypothesis was that the Th2 pathway may counteract protective Th-1 immunity.

2.4.2.1.5 Immunology of feline URT aspergillosis

No studies have been performed to determine the immune-response of SNA and SOA in cats.

2.4.2.2 Epidemiology

In both cats and dogs URTA typically occurs in young to middle-aged animals. Of 25 reported feline cases the mean age at diagnosis was 7.5 years with a range from 1.9 to 13 years (Barachetti et al., 2009; Furrow and Groman, 2009; Giordano et al., 2010; Goodall et al., 1984; Halenda and Reed, 1997; Hamilton et al., 2000; Kano et al., 2008; Karnik et al., 2009; Malik et al., 2010; Malik et al., 2004; McLellan et al., 2006; Peiffer et al., 1980; Smith and Hoffman, 2010; Tomsa et al., 2003; Whitney et al., 2005). In case-series of canine SNA, mean age at diagnosis was 5 to 6 years (Billen et al., 2010; Billen et al., 2009; Johnson et al., 2006; Pomrantz and Johnson, 2010; Pomrantz et al., 2007;

Saunders et al., 2002; Sharman et al., 2010) Males appear to be over-represented in canine SNA and possibly in feline URT aspergillosis (16 of 24 cases where gender was specified). In combined reports of 377 cases of canine SNA there were 245 males and 132 females with a male to female ratio of 1.9:1 (Billen et al., 2010; Billen et al., 2009; De Lorenzi et al., 2006; Johnson et al., 2006; Mathews et al., 1998; Pomrantz and Johnson, 2010; Pomrantz et al., 2007; Saunders et al., 2002; Sharp et al., 1991a; Zonderland et al., 2002).

A striking difference between feline and canine URTA is the facial conformation of affected animals. As more cases of feline URTA are reported there is an apparent over-representation of brachycephalic breeds, with 10 of 24 cases being of Persian or Himalayan breed (Himalayans are essentially colour-variants of the Persian breed) (Barachetti et al., 2009; Hamilton et al., 2000; Karnik et al., 2009; Malik et al., 2004; McLellan et al., 2006; Tomsa et al., 2003; Whitney et al., 2005). By contrast dolicocephalic and mesaticephalic breeds of dogs are predisposed to SNA and infection in brachycephalic breeds is rare. In most case-series comparisons with hospital populations to calculate Odd's ratios were not performed, thus overt breed predispositions have not been confirmed. However, breeds repeatedly identified include Rottweilers, Labrador Retrievers, Golden Retrievers and German Shepherd dogs (Billen et al., 2010; Billen et al., 2009; Mathews et al., 1998; Pomrantz et al., 2007; Saunders et al., 2002; Zonderland et al., 2002)

The reasons why brachycephalic cats and dolicocephalic or mesaticephalic dogs are predisposed to URTA remain elusive. There is no evidence of an association between retrovirus infection and URT aspergillosis, with only one FeLV positive case reported (Goodall et al., 1984). Most dogs with canine SNA are systemically healthy. Reduced drainage of URT secretions due to brachycephalic conformation alone could be a risk factor for aspergillosis in cats. However, since brachycephalic dogs are under-represented for SNA, it is likely that additional risk factors are present in cats. These

could include heritable defects in mucosal immunity, previous viral URT infection and previous antibiotic treatment favouring fungal colonisation (Tomsa et al., 2003).

2.4.2.3 Clinical presentation in dogs and cats

In dogs the triad of muzzle pain, profuse mucopurulent to haemorrhagic chronic nasal discharge and depigmentation, crusting or ulceration of one or both nares is highly suggestive of SNA. Infrequently facial deformity and/or seizures may occur. Presenting signs in cats with SNA are more subtle and include a history of sneezing, unilateral or bilateral serous to mucopurulent nasal discharge and intermittent epistaxis in approximately 40% of cases (Furrow and Groman, 2009; Goodall et al., 1984; Quimby et al., 2010; Tomsa et al., 2003; Whitney et al., 2005). In one case a discharging sinus was identified overlying the frontal sinus (Malik et al., 2004). Nasal depigmentation or ulceration has not been documented in cats with SNA. Cats with SOA are usually presented for investigation of unilateral exophthalmos. Clinical signs are referable to invasive expansion of a fungal granuloma in the ventromedial orbit. In addition to exophthalmos, these include dorsolateral deviation of the globe, conjunctival hyperemia, third eyelid prolapse and exposure keratitis (Barachetti et al., 2009; Giordano et al., 2010; Halenda and Reed, 1997; Hamilton et al., 2000; McLellan et al., 2006; Smith and Hoffman, 2010; Wilkinson et al., 1982). In some cases an oral cavity mass in the ipsilateral pterygopalatine fossa behind the last molar tooth has been identified, reflecting ventral extension of the fungal granuloma from the orbit. Extension of infection outside the sinonasal cavity may cause facial distortion, including swelling of the nasal bridge, periorbital tissues and soft tissues adjacent the maxilla. At the time of presentation, nasal signs may be absent (Smith and Hoffman, 2010). Neurological signs occur when there is CNS invasion or involvement of both optic nerves and the optic chiasm and include blindness and seizures (Barachetti et al., 2009; Giordano et al., 2010; Smith and Hoffman, 2010).

2.5 Diagnosis of aspergillosis

2.5.1 Serology and antigen detection in tissues and/or body fluids

Serological tests have important applications as early non-invasive diagnostic tests for aspergillosis in humans and companion animals. Tests include detection of serum anti-*Aspergillus* antibodies and detection of *Aspergillus* antigens in serum, body-fluids or tissue. Structurally, the fungal cell wall consists of structural polysaccharides, primarily chitin and glucans, organized into microfibril bundles and an amorphous component, primarily glycoproteins and other polysaccharides that cover the inner microfibrillar layer. Chitin and glucans provide rigidity for the cell wall and prevent osmotic distension of the fungal cell. β-glucans are the most abundant structural polysaccharides and are composed of glucose residues in 1-3-β linkages or 1-6-β linkages. Mannan is the most abundant of the amorphous polysaccharides (Sentandreu et al., 2004).

2.5.1.1 Galactomannan detection

Galactomannan (GM) is a water soluble, heat-stable polysaccharide outer cell wall component of *Aspergillus* species that is continuously and actively secreted during mycelial growth. It is released into the circulation during hyphal invasion into tissue. GM consists of a mannan core and galactofuran side chains (Latge, 2009). Methods for measuring GM in serum and body fluids or tissues include a sandwich ELISA immunoassay (Platelia™ *Aspergillus* EIA, BioRad, Marnes-la-Coquette, France) and a latex agglutination test (Pastorex *Aspergillus* latex agglutination (LA) test (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). The ELISA has largely replaced the latex agglutination test due to its lower limit of detection of GM (1 ng/L compared to 15 ng/L) (Verweij et al., 1995). The Platelia ELISA utilizes a rat monoclonal antibody (EB-A2) that binds to the galctofuran epitope of the GM antigen, specifically to the (1→5)-β-D-galactofuranosyl (gal*f*) side chains (Stynen et al., 1995; Swanink et al., 1997). In addition to GM, the culture supernatant of *A. fumigatus* contains several glycoproteins that also react with the EB-A2 antibody (Latge, 2009).

2.5.1.1.1 Galactomannan detection - humans

Serum GM detection is a non-invasive test that yields rapid results in critically ill patients not suited for other diagnostic procedures that could give definitive test results, such as CT-guided lung biopsy or fine-needle aspirate. Detection of GM in human patients with invasive aspergillosis (IA) has the highest sensitivity in immunocompromised patients, especially in neutropenic patients (Cordonnier et al., 2009). In non-neutropenic patients the sensitivity of the Platelia ELISA is very low. For example, in a meta-analysis of GM performance by patient population (including 27 studies and 4284 confirmed cases of IA) the sensitivity (22%) and specificity (84%) of the assay was lowest in solid organ transplant patients, who are usually not neutropenic (Pfeiffer et al., 2006). Low sensitivity of the assay in immunocompetent patients is partly associated with antigen clearance by neutrophils, which possess mannose-binding receptors, or by complexing via circulating anti-*Aspergillus* antibodies (Herbrecht et al., 2002; Mennink-Kersten et al., 2004). Other causes of false negative results in patients with IA using the Platelia ELISA include low fungal burdens, prior or concomitant antifungal therapy and walled off infections (Hsu et al., 2011).

In addition to poor sensitivity in certain patient cohorts, the diagnostic utility of serum GM is hampered by poor specificity in other patient cohorts. Risk factors for false-positive results include patients treated with β-lactam antibiotics such as amoxicillin-clavulanate or with the sodium-gluconate containing intravenous crystalloid rehydration fluid Plasmalyte (Racil et al., 2007; Zandijk et al., 2008). False-positive results are due to contamination with small amounts of GM produced by fermentation of *Aspergillus niger* during synthesis of these medical products (Hage et al., 2007). In addition false positive results are reported in paediatric patients, reportedly due to absorption of dietary GM across an immature or impaired gastrointestinal epithelium (Herbrecht et al., 2002), and in patients with cryptococcosis due to cross-reactive epitopes on the *C. neoformans* antigen galactoxylomannan that mimic *Aspergillus* GM (Dalle et al., 2005).

Other “false-positive” results can occur with non-Aspergillus filamentous fungal infections. GM has been detected in many other filamentous fungi, including *Penicillium*, *Paecilomyces*, *Botrytis, Fusarium, Altenaria, Cladosporium, Trichophyton, Geotrichum* and *Wangiella*, albeit at lower levels than for *Aspergillus* species (Quindos, 2006; Swanink et al., 1997).

More recently, the Platelia ELISA has been utilised to detect GM in bronchoalveolar lavage fluid (BAL) in patients at risk of IA (Acosta et al., 2011; D'Haese et al., 2012; Meersseman et al., 2008; Nguyen et al., 2007). Overall, this method of GM detection has is significantly more sensitive than GM detection in serum and has an excellent diagnostic accuracy provided results are interpreted in parallel with clinical and radiological findings and with consideration of pre-test probabilities. In some studies the sensitivity of BAL GM was higher than that for fungal culture and/or microscopy (Meersseman et al., 2008), whilst in others the sensitivity of culture and/or microscopy and GM in BAL were both high (Nguyen et al., 2007). Regardless, one advantage of BAL GM detection is the rapid diagnostic result, with a turnaround time of hours compared to several days for positive fungal culture. However, consideration must be given to false positive results, with the same risk factors as for serum GM detection, to avoid unnecessary treatment of critically ill patients with potentially toxic systemic antifungal drugs (Boonsarngsuk et al., 2010; Hage et al., 2007).

2.5.1.1.2 Galactomannan detection - dogs

The Platelia ELISA was evaluated for diagnosis of SNA in dogs in a study of 17 dogs with SNA, 18 dogs with nasal cancer, 11 dogs with inflammatory (lymphoplasmacytic) rhinitis and 33 control dogs (Billen et al., 2009). Overall sensitivity was 24% and specificity was 82%. Twenty four percent of dogs with SNA tested positive, as did 11% of dogs with nasal tumours, 9% of dogs with non-fungal rhinitis and 24% of healthy dogs. The low sensitivity of serum GM for diagnosis of canine SNA is perhaps not surprising, since in contrast to IA in people, canine SNA in dogs is a non-invasive

mycosis in which fungal hyphae colonise the surfaces of the sinonasal epithelium but do not penetrate the respiratory mucosa (Peeters et al., 2005). Likely factors contributing to this low sensitivity include the absence of angioinvasion, as well as antigen clearance or binding in these systemically immunocompetent dogs. By contrast, GM is often detected in the serum of dogs with systemic mycoses (Garcia et al., 2011).

Interestingly, one study of FRS in humans evaluated the use of GM detection in the supernatant of sinonasal pus or fungus ball diluted in saline then centrifuged. The sensitivity and specificity of the Platelia ELISA were 87% and 88% respectively (Kauffmann-Lacroix et al., 2001). A similar study, utilising nasal lavage fluid for GM detection in patients with FRS did not find repeatable results – 7 patients with FRS were all negative in the GM ELISA (Kostamo et al., 2007), while 21% of control patients tested positive. Despite the disparity in results between these two studies, investigation of GM detection in nasal lavage fluid from dogs with SNA warrants further investigation.

2.5.1.1.3 Galactomannan detection - cats

The diagnostic utility of serum GM detection was recently evaluated in cats with URT aspergillosis ((Whitney et al., 2013a). The Platelia ELISA was used to detect serum GM in 13 cats with confirmed URT aspergillosis including 6 with SNA and 7 with SOA (Group 1). Control groups included 15 cats with non-aspergillosis URT disease (Group 2), 14 cats treated with β-lactam antibiotics for non-respiratory tract disease (Group 3), 31 healthy young cats < 2 y of age (Group 4b) and 13 healthy adult cats ≥ 2 y of age. One cat with SNA and two cats with SOA caused by an *Aspergillus fumigatus*-mimetic species, tested positive for serum GM. The overall sensitivity and specificity of the assay was 23% and 78% respectively. Similar to human studies, false positive results were common in young cats (29%) and in β-lactam antibiotic-treated cats (32%). The specificity of the assay increased to 93% when these known high risk false positive groups were excluded from the analysis. In both cats and dogs with URT aspergillosis, serum GM testing cannot be recommended as a routine diagnostic test.

2.5.1.2 Serum 1-3-B-D-glucan detection

Unlike GM, which has diagnostic specificity for aspergillosis, 1-3-β-D-glucan (BG) present in the fungal wall of most fungal species with the notable exception of the zygomycetes (*Mucor* spp.). *Cryptococcal* spp. exhibit no, low-level of variable BG expression. Detection of BG in serum or BAL fluid is a panfungal diagnostic test for detection of invasive fungal infections in people. A limitation of its use is the lack of ability to discriminate between fungal general and species which compromises the ability to select the most appropriate antifungal agent. Commercial assays developed to measure 1-3-β-D-glucan detect its ability to activate a coagulation cascade within horseshoe crab haemolymph (Estrella et al., 2011; Thornton, 2010). These assays are able to detect BG concentrations approaching 1 pg/ml. Very few studies have evaluated the utility of BG detection in serum or body fluids from patients with IA compared to GM detection. In a recent study of ICU patients with fungal pneumonia predominantly due to IA, using the optimal cut-off for detection based on receiver operating curve analysis of > 80 pg/ml, the sensitivity and specificity of BG detection in serum was >80% and the negative predictive value was high (93%) (Acosta et al., 2011). It has been suggested that BG and GM should be used together as markers for invasive fungal infections (IFI) (Hachem et al., 2009). Where both BG and GM were evaluated simultaneously in patients with IFI BG had a higher sensitivity than that of GM for detecting IA and other mold infections in patients with haematologic malignancy, but had a lower specificity (Hachem et al., 2009). Risk factors for false positive BG results include bacteraemia, treatment with β-lactam antibiotics, haemodialysis with cellulose products, cardiopulmonary bypass, immunoglobulin or albumin infusions and exposure to glucan-containing gauze during surgery (Hope et al., 2005b; Thornton, 2010). Serum BG detection has not been evaluated for the diagnosis of URT aspergillosis in dogs or cats.

2.5.1.3 Serum antibody detection - humans

Detection of anti-*Aspergillus* antibodies in the serum of patients with IA was proven to be unreliable for diagnosis, since only a third or less of affected patients mounted a detectable antibody response (Herbrecht et al., 2002; Hope et al., 2005b; Kappe et al., 1996). Aspergillins used in early assays consisted of crude somatic antigen extracts from *A. fumigatus* hyphal biomass, galactomannan or other metabolic antigens and a wide range of assay formats were used including immunodiffusion, counter immunoelectrophoresis (CIE), complement fixation, haemagglutination assays, indirect-immunofluorescence, radioimmunoassay and ELISA (Kappe et al., 1996). The majority of patients with IA are unable to mount an antibody response to these *Aspergillus* antigens because of immune suppression or malignancy. The development of other non-invasive diagnostic tests for IA was focused on antigen detection subsequently, since sensitivity of antigen detection was higher in this cohort of patients. For smaller subsets of immunocompetent patients with chronic pulmonary aspergillosis (CPA) antibody detection has proved to be a more useful diagnostic tool than GM or BG detection. For instance, in a recent study of patients with CPA, the sensitivity and specificity of an *Aspergillus* precipitating antibody (immunodiffusion) test was 74% and 84% respectively (Ohba et al., 2012). In another study of CPA patients, serum *Aspergillus* precipitating antibody positivity was 89%, compared to 50% for GM detection and 15% for BG detection (Kitasato et al., 2009).

More recently the use of purified recombinant antigen preparations containing single proteins or polysaccharides has been investigated for serological diagnosis of IA. One such protein, recombinant *A. fumigatus* mitogillin, expressed in *E. coli*, was found to improve the sensitivity of serological diagnosis of IA by detection of specific anti-mitogillin antibodies. Positive IgG titres were detected in the sera of 64% (27 of 42) patients with IPA, but in only 1% of healthy controls (Weig et al., 2001).

2.5.1.4 Serum antibody detection – dogs and cats

Serum anti-*Aspergillus* antibodies can be detected by numerous methods including counter-immunoelectrophoresis (CIE), agar gel immunodiffusion (AGID) or ELISA.

These tests have been applied to individual cases of feline URTA only, thus the sensitivity and specificity of antibody detection for diagnosis of feline aspergillosis is currently not known. Five of 10 reported cases (9 SNA, 1 SOA) tested seropositive (Barrs et al., 2012a; Furrow and Groman, 2009; Goodall et al., 1984; Tomsa et al., 2003; Whitney et al., 2005). By contrast, two recent studies evaluated serology for diagnosis of canine SNA (Billen et al., 2009; Pomrantz et al., 2007). In one study AGID was compared with fungal culture of nasal biopsies (Pomrantz et al., 2007), and in the other the AGID and ELISA were compared (Billen et al., 2009). For both methods a purified aspergillin preparation composed of extracts of *A. fumigatus*, A. *niger* and *A. flavus* was used. In one study fungal culture was more sensitive (81%) than serologic testing (67%) while specificity was high for both fungal culture (100%) and serology (98%) (Pomrantz et al., 2007). In the second study ELISA had higher sensitivity (88%) than AGID (76%) and specificity was high for both methods (ELISA 97%, AGID 100%) (Billen et al., 2009). These studies demonstrate that seropositivity for *Aspergillus* spp. is highly suggestive of SNA in dogs but that negative test results do not rule out aspergillosis.

2.5.2 Molecular methods for diagnosis of aspergillosis in cats, dogs and humans

For species recognition in the clinical setting comparative sequence based methods used in conjunction with traditional phenotype based methods offers a practical approach to resolution of species within the genus *Aspergillus* (Balajee et al., 2007). Methods for identification of fungi from clinical specimens include conventional PCR and sequencing of DNA extracted from fungal cultures as described in section 1.2.5, or PCR of DNA extracted directly from clinical specimens. Depending on the type of clinical specimen, conventional PCR or real time quantitative PCR (qPCR) can be performed. A major reason for utilization of qPCRs over conventional PCRs in routine diagnostic mycology laboratories for diagnosis of aspergillosis in humans is the decreased risk of amplification of aerosolized fungal DNA contaminating the environment, since amplification and detection take place in the same tube without the need to open it

(Bretagne, 2011). In order to take precautions to rule out false negative results in qPCR assays standardization of assays is important. This includes the routine incorporation of an internal amplification control (IAC) to assess for presence of PCR inhibitors in the sample (for example haeme and cells in blood or BAL fluid) and an extraction control to confirm successful DNA extraction in the sample. To assess for PCR inhibition each sample can be spiked with an exogenous IAC, for example the jellyfish aequorin gene. PCR is then performed to detect the aequorin gene in the spiked sample. If amplification does not occur then inhibition is present and DNA extraction should be reattempted from the original sample (Khot et al., 2008). Examples of DNA extraction controls include the GAPDH gene in dogs and cats (Windsor et al., 2006) and the 18s rRNA gene in humans (Khot et al., 2008). False positive results can occur due contamination with environmental fungi during sample collection, DNA extraction and PCR set-up, or from fungal PCR product carry-over. In addition, false positives can occur in the setting of suboptimal analytical specificity in the qPCR, resulting from cross-reactivity of the target qPCR assay with other (nontarget) fungi or DNA (Khot et al., 2008).

2.5.2.1 Molecular methods using formalin fixed paraffin embedded tissues (FFPET) - humans

In situations where fungal elements are seen in histological specimens but fungal culture was either negative or not performed, molecular methods are useful to identify the infecting fungus. One of the limitations of utilizing FFPET is that they often only yield short DNA fragments, thus limiting the gene targets for amplification. To overcome this issue probe and primers designed for known pathogens can confirm the identification, but only for the predefined species. A conventional panfungal PCR targeting the ITS1 region and designed to amplify a 361 base-pair product was reported to successfully identify the fungal pathogen in 64% of histologically proven invasive fungal infections in one study (Lau et al., 2007).

Another study compared conventional PCRs amplifying the ITS1 and ITS2 regions, both designed to amplify products of > 300 base pairs, with qPCR amplifying *Aspergillus* spp. mitochondrial DNA, designed to amplify a product of < 150 base pairs in patients with confirmed sinus fungal balls (a non-invasive form of FRS) (Cabaret et al., 2011). ITS1 or ITS2 sequencing identified the aetiologic agent in 10 of 16 cases (*A. fumigatus* (n=9), *Lewia* spp. (n=1)). The size of the sequences amplified ranged from 120 to 352 base pairs. The qPCR amplified *Aspergillus* spp. DNA in 15 of the 16 cases (sequencing of the qPCR products was not performed). The negative sample was the *Lewia* spp. This study illustrates the benefits of qPCR for amplification of short fungal targets for predefined species confirmation without the need for sequencing but also illustrates the benefits of a panfungal PCR to identify unexpected or unknown fungal pathogens.

Using qPCR and sequencing of the ITS2 and 28S (large subunit) regions of the rDNA gene complex (Figure 4), investigators correctly identified fungal pathogens in 80% of cases of proven invasive septate mould infections (Rickerts et al., 2011). To minimise inadvertent amplification of human rDNA in FFPET biopsies, modified primers were used that maximized the number of nucleotide mismatches with the human rDNA gene. The primers utilized to amplify the ITS2 region were 5.8S forward and 28S-1 reverse, and for the 28S regions were 28-S10 forward and 28S-12 reverse (Khot et al., 2009).

2.5.2.1.1 Molecular methods using FFPET- dogs and cats

Conventional ITS1 PCR was utilized for identification of fungal pathogens in one cat with SOA using FFPET from a retrobulbar mass. The fungal pathogen had been identified as *A. fumigatus* on culture while the amplified ITS1 sequences had 100% homology with GenBank sequences of *Neosartorya pseudofischeri* (Lau et al., 2007). This was the sentinel case that sparked my interest in feline aspergillosis as I had referred the clinical specimen from this cat to these investigators to assist in validation of their PCR assay for molecular identification of fungi in FFPET. This was the first case to demonstrate molecular identity of SOA in cats and subsequently resulted in

collaboration with the same investigators to pursue the aetiology of URT aspergillosis in cats (see Chapter three). The application of qPCR for identification of fungal pathogens in dogs with SNA using FFPET is discussed in section 2.3 and below.

2.5.2.2 Real time quantitative PCR (qPCR) using clinical specimens

Quantitative PCR is highly sensitive with the potential to detect a few gene copies per reaction or less than a single genome for multicopy genes such as the rDNA gene complex. Since qPCR can measure the amount of microbial DNA in a clinical sample it also has the potential to discriminate between colonization and infection. The nasal cavities of cats and dogs can be transiently contaminated or colonized by fungal species, as demonstrated by studies evaluating presence of *Cryptococcus* spp. in cats and dogs with negative serum latex agglutination test titres (Duncan et al., 2005a, b). *Aspergillus* spp. colonization of the nasal cavity in dogs and cats has not been determined by culture studies. Using a genus specific qPCR to detect ribosomal DNA from all *Penicillium* and *Aspergillus* spp. fungal DNA was detected in nasal biopsies of dogs with SNA as well as in all the control dogs in that study, demonstrating that fungi are likely constituents of the normal flora of the nasal cavity of the dog (Peeters et al., 2008). Using another rDNA panfungal qPCR to detect fungi in nasal biopsies (FFPET) it was demonstrated that dogs with SNA had significantly higher fungal DNA loads than dogs with neoplasia and dogs with chronic lymphoplasmacytic rhinitis (LPR). They also demonstrated that dogs with LPR had higher fungal DNA loads than dogs with nasal neoplasia, suggesting a causal relationship between the inflammation seen in LPR and fungal colonization of the nasal cavity (Windsor et al., 2006).

A number of different qPCR assays have been developed to detect fungal DNA in blood or BAL fluid of human patients with IA. The assays include a commercial MycAssay (Myconostica Ltd) developed for the detection of genomic DNA from 15 different species of *Aspergillus*, including the 4 most common human pathogens (*A. fumigatus, A. flavus, A. terreus* and *A. niger).* The target is a 114bp segment of the 18S rDNA gene

and the assay includes an internal amplification control (IAC) of plant origin to detect any inhibitory substances in the sample. A similar pan-Aspergillus qPCR targeting the 18s rDNA region is specific for over a 100 *Aspergillus* species (Walsh et al., 2011). However, it should be noted that these pan-Aspergillus PCRs will amplify *Penicillium* spp. and occasionally *Paecilomyces* spp. (Walsh et al., 2011).
Other pan-Aspergillus or pan-fungal qPCR assays have been developed targeting the ITS regions and 28S rDNA regions of the rDNA gene complex (Khot et al., 2009; Khot et al., 2008; Rickerts et al., 2011). Species-specific qPCRs targeting the ITS1 regions of the major *Aspergillus* spp. pathogens have also been developed (Walsh et al., 2011). Although molecular identification of *Aspergillus* spp. is most accurate using primers and probes targeting two or three gene loci, species-specific qPCRs can be used in clinical settings alongside genus-specific qPCRs to provide additional information for targeted antifungal therapy.

Quantitative PCR is becoming increasingly utilized as a diagnostic test to detect fungi in BAL fluid from patients with IPA and has comparable performance to GM measurement (Torelli et al., 2011). In a clinical setting, fungal DNA detection by qPCR can be combined with GM and culture results to increase diagnostic accuracy (Walsh et al., 2011). Also, *Aspergillus* colonization can be distinguished from invasive infection to some extent. In one study where the optimal cut-off threshold cycle value (Ct) of ≤ 35 was calculated using receiver operating curve (ROC) analysis, all patients with IPA had Ct values of ≤ 35 compared with 50% of patients with *Aspergillus* colonization of the airways (Luong et al., 2011).

2.5.3 Diagnostic imaging

Computed tomography (CT) has virtually replaced conventional radiography for the assessment of nasal cavity and orbital disease in dogs and cats. CT was found to be more sensitive than plain radiology in the diagnosis of SNA in dogs in two studies, with a sensitivity of 88-92% compared with 72-84% for plain radiography (Saunders and Van

Bree, 2003; Saunders et al., 2002). The advantages of CT over radiography include increased sensitivity of diagnosis, elimination of superimposition of structures through cross-sectional imaging, and multiplanar reconstructions which allow better evaluation of skull structures including the cribriform plate and orbit. Imaging studies should be performed before rhinoscopy and biopsy since iatrogenic haemorrhage can induce imaging abnormalities and obscure subtle lesions (Saunders et al., 2004). Magnetic Resonance Imaging (MRI) is also useful for diagnosis of aspergillosis in dogs and cats and is generally superior to CT for imaging of soft-tissue changes and inferior for detection of changes in cortical bone. In one small prospective study MRI and CT were both more sensitive than radiography in detecting changes diagnostic of SNA (Saunders et al., 2004) Generally, MRI is indicated over CT where there is CNS involvement (Giordano et al., 2010).

2.5.3.1 Computed tomographic findings in canine SNA

CT findings in dogs with SNA have been well characterised and most commonly include:

(i) cavitated-like turbinate lysis
(ii) a rim of soft-tissue of variable thickness along bones in the frontal sinus, maxillary recess and nasal passages. The rim of soft-tissue is due to thickening of the mucosa adjacent the inner surfaces of these bones. **(Fig 6a)**
(iii) Abnormal presence of soft-tissue accumulation **(Fig 6b)**
(iv) Thickened, reactive bony-changes – hyperostosis (**Fig 6c)**

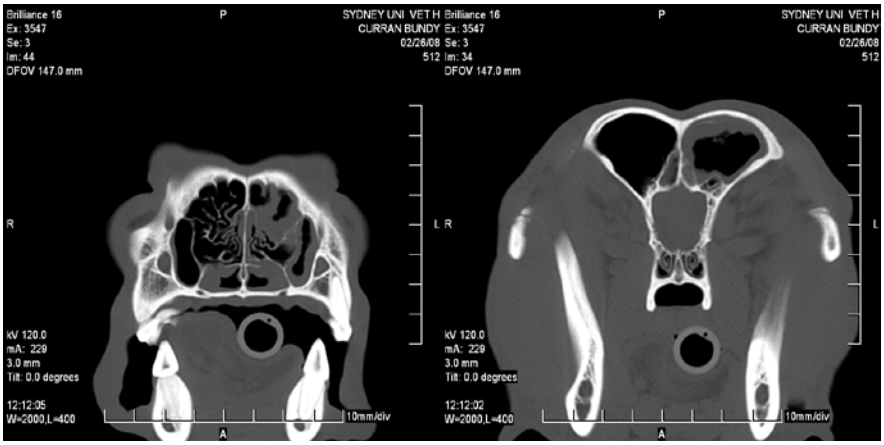


Fig 6a (i) (left) and 6a (ii) (right). On these transverse CT images of the skull of a 4-year old male desexed cattle x kelpie dog that presented to the University Veterinary Teaching Hospital, Sydney with sinonasal aspergillosis affecting the left side of the nasal cavity and frontal sinuses. There is an accumulation of abnormal soft tissue in the left side of the nasal cavity including a thickened rim of soft-tissue in the maxillary recess **(Fig 6 a (i))** and a rim of thickened tissue adjacent the inner surface of the left frontal sinus bone (**Fig 6a (ii)**. *Images courtesy University Veterinary Teaching Hospital, Sydney.*

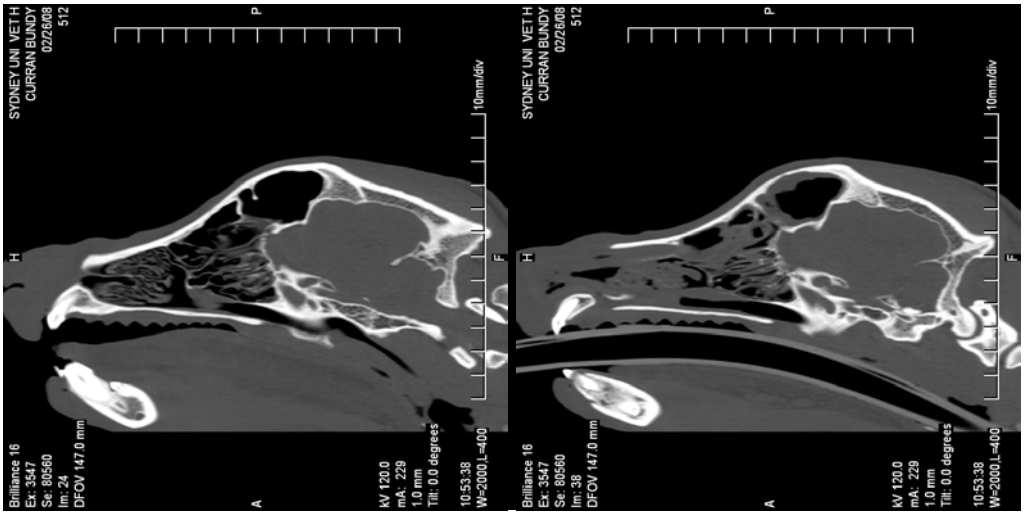


Fig 6b (i) (left) and 6b (ii) (right): Sagittal skull CT images from the dog in **Fig 6b (i) –** On this image, taken to the right of the midline the nasal turbinates are intact and soft tissues appear normal. The frontal sinus is also normal. **Fig 6 b (ii)** On this image, taken to the left of the midline there is an accumulation of abnormal soft tissue and some turbinate lysis in the rostral nasal cavity. Also, there is a thickened rim of soft-tissue around the frontal sinus. *Images courtesy University Veterinary Teaching Hospital, Sydney.*

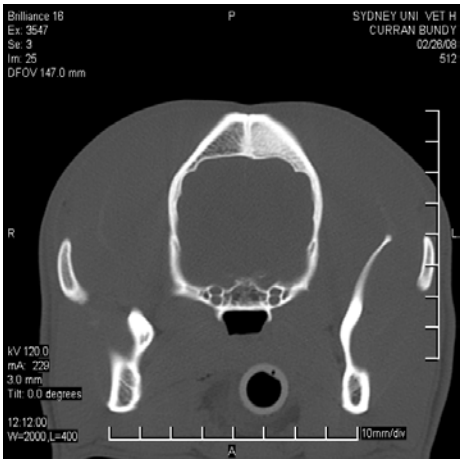


Fig 6c. Transverse CT image of the skull of the same dog in **Fig 6a** taken at the level of the frontal sinuses. The bone of the left frontal sinus is hyperostotic (increased radiodensity). *Image courtesy University Veterinary Teaching Hospital, Sydney.*

2.5.3.2 Computed tomographic findings in feline URT aspergillosis

CT findings have been described in case reports of 11 cats with SNA (Furrow and Groman, 2009; Karnik et al., 2009; Tomsa et al., 2003; Whitney et al., 2005) and in 10 cats with SOA (Barachetti et al., 2009; Giordano et al., 2010; Halenda and Reed, 1997; Hamilton et al., 2000; Kano et al., 2008; Karnik et al., 2009; McLellan et al., 2006; Smith and Hoffman, 2010). Although CT changes in cats were less specific than those documented in dogs with SNA similarities included turbinate lysis and abnormal soft-tissue accumulation within the nasal passages (Furrow and Groman, 2009; Whitney et

al., 2005). Involvement of the nasal cavity was mostly bilateral (Furrow and Groman, 2009; Karnik et al., 2009) and punctuate lysis of the orbital lamina and palatine bone was sometimes observed (Karnik et al., 2009; Whitney et al., 2005). In one report there was a calcified discrete density in the middle right nasal cavity presumed to be necrotic tissue that had undergone dystrophic calcification (Tomsa et al., 2003). Calcification of fungal plaques due to deposition of calcium oxalate or phosphate crystals, thought to be fungal metabolites, occurs in sinonasal *Aspergillus sp*. infections in humans, in approximately 50% of noninvasive Aspergillus sinus fungal balls (mycetomas) with maxillary sinus involvement (Lenglinger et al., 1996).

Of the 10 reported cases of feline SOA where CTs were performed, the most common findings were a unilateral ventromedial orbital mass resulting in dorsolateral displacement and sometimes indentation of the globe, nasal cavity involvement and punctuate lysis of the frontal bone (orbital lamina or floor of frontal sinus) (Barachetti et al., 2009; Giordano et al., 2010; Halenda and Reed, 1997; Hamilton et al., 2000; Kano et al., 2008; Karnik et al., 2009; McLellan et al., 2006; Smith and Hoffman, 2010). Soft-tissue attenuation and heterogeneous enhancement post-contrast of the orbital mass was also reported (Giordano et al., 2010; Karnik et al., 2009; Smith and Hoffman, 2010). Features of SOA that overlap those seen in nasal neoplasia included soft tissue mass effect and osteolysis.

2.5.4 Histology and Immunohistochemistry

2.5.4.1 Canine SNA

Histologic and immunohistochemical features of canine SNA were characterised in a study of 15 affected dogs (Peeters et al., 2005). The most common findings included an ulcerated and severely inflamed mucosa, often covered by a plaque of necrotic tissue. Fungal infection was non-invasive; fungal hyphae were most commonly present in necrotic plaques adjacent the mucosa or within luminal exudates but did not penetrate

the mucosal epithelium. Fungal hyphae were directly associated with the mucosal surface in two of 15 cases only. The underlying lamina propria was heavily infiltrated by mixed mononuclear inflammatory cells in a densely packed sheet disrupting the normal mucosal structure. These cells consisted mostly of CD4+ and CD8+ T-lymphocytes and IgG+ plasma cells, with fewer MHC II+ activated antigen-presenting cells (macrophages and dendritic cells). L1+ macrophages and neutrophils were also present in the lamina propria and epithelium, consistent with active recruitment of myelomonocytic lineages from the blood. The immunohistological findings in dogs with SNA were consistent with a dominant Th1-regulated cell mediated immune response effective in preventing systemic dissemination of the fungus but ineffective in clearing the infection from the nasal cavity and frontal sinus (Day, 2009; Peeters et al., 2005).

2.5.4.2 Feline URT aspergillosis

Histological findings the sinonasal form of feline URT aspergillosis have not been well characterised and immunohistochemical studies have not been described for either form. Histologic changes on nasal biopsies reported for feline SNA include severe inflammatory rhinitis with lymphoplasmacytic or mixed-cell inflammatory cell infiltrates, necrosis, which can be extensive, and mats of fungal hyphae (Furrow and Groman, 2009; Tomsa et al., 2003; Whitney et al., 2005).

Histopathological changes have been described in seven cases of feline SOA (Barachetti et al., 2009; Giordano et al., 2010; Hamilton et al., 2000; McLellan et al., 2006; Smith and Hoffman, 2010). Key findings include a propensity to invade contiguous anatomic planes including the nasal cavity, orbital tissues external to the globe and nictitating membrane. The development of CNS signs in some cases implies fungal invasion of CNS tissue, however brain histology was not performed in the two cats in which seizures were described (Giordano et al., 2010; Smith and Hoffman, 2010). In one case with bilateral orbital granulomas and complete loss of vision histological changes were present in the optic chiasm which exhibited diffuse, moderate

gliosis and spongiosis and a mild lymphocytic–plasmacytic meningitis. However, fungal hyphae were not identified within the optic chiasm or other areas of the CNS (Barachetti et al., 2009). In cats with SOA orbital granulomas are typically comprised of multifocal to coalescing granulomas with abundant areas of coagulative necrosis in which septate fungal hyphae are present. Central areas of coagulative necrosis are surrounded by zones of eosinophils, macrophages, lymphocytes and fibroplasia.

2.6 Treatment and Prognosis

2.6.1 Treatment of canine SNA

In general, the prognosis for treatment of canine SNA is good, but treatment can be challenging. Since canine SNA is a noninvasive mycosis, topical therapy is considered more effective than systemic therapy because of direct contact with fungal plaques, although no large scale studies have evaluated the efficacy of suitable systemic antifungal medications such as itraconazole (Peeters and Clercx, 2007). Topical azole antifungals, including 1% clotrimazole, 1% enilconazole or 1% bifonazole are delivered intranasally or via sinus trephination. However, multiple applications are often required to achieve clinical cure. In a multicentre, retrospective study of 81 dogs with SNA treated with topical clotrimazole or enilconazole using catheters placed intranasally and/or via sinus trephination, a single treatment was successful in 47% (Sharman et al., 2010). Similarly, in a smaller prospective study of 23 dogs with SNA treated with a 1% clotrimazole intranasal infusion, infection resolved in 48% of cases after one treatment (Pomrantz and Johnson, 2010). Techniques reported to improve efficacy of topical antifungals include endoscopic/sinuscopic debridement of sinonasal fungal plaques prior to therapy (Billen et al., 2010; Zonderland et al., 2002), endoscopic guidance of infusion catheters into the caudal frontal sinus (Saunders et al., 2002) and use of depot preparations of 1% clotrimazole or 1% bifonazole cream (Billen et al., 2010).

In a canine cadaver study, clotrimazole cream (1% depot preparation) persisted in the frontal sinuses 96 h after installation via sinus trephination compared with 1 minute for a

clotrimazole substitute solution in propylene glycol, demonstrating a possible advantage of increased contact time with fungal plaques using the depot preparation (Hayes and Demetriou, 2012). The same study also showed that distribution of a 50 ml infusate of a clotrimazole substitute solution instilled via sinus trephination of the lateral frontal sinus resulted in inadequate coating of the rostral compartment of the frontal sinus, whereas medicating both the lateral and rostral compartments either by double trephination or a single oblique trephination resulted in complete coating of all frontal sinus mucosa. In cases of canine SNA where there is involvement of the rostral frontal sinus as indicated by advanced diagnostic imaging, consideration should be given to medicating both rostral and lateral compartments of the frontal sinus. Adverse effects reported in association with topical antifungal therapies in the treatment on canine SNA include seizures (Billen et al., 2010) and severe pharyngeal oedema (reference). Seizures are thought to be the result of direct contact of intranasally instilled antifungal preparations with the CNS where fungal infection has resulted in lysis of the cribriform plate (Billen et al., 2010). Assessment of the integrity of the cribriform plate by CT examination before treatment is recommended.

2.6.2 Treatment of feline URT aspergillosis

Information on treatment of feline SNA is limited to several case reports (Furrow and Groman, 2009; Goodall et al., 1984; Tomsa et al., 2003; Whitney et al., 2005). Treatment was successful in most cases using treatment regimens including systemic antifungal therapy alone, systemic triazole therapy combined with topical intranasal clotrimazole or topical therapy alone. Anatomical landmarks have been defined for sinus trephination in cats (Winstanley, 1974). Trephine openings are made slightly to the side of the mid-line on a line that joins the anterior borders of the supra-orbital processes.

Similar to canine SNA, the importance of debridement of fungal plaques is illustrated by resolution of signs in one case after rhinoscopy and sinonasal cavity lavage (Tomsa et

al., 2003) and in another after sinusotomy/rhinotomy and instillation of iodoform paste (Goodall et al., 1984). Presence or absence of orbital involvement in cats with URTA has important prognostic significance. Few cases of SOA have been treated successfully despite aggressive therapy including orbital exenteration and use of newer generation fungicidal triazoles (posaconazole or voriconazole), liposomal amphotericin-B and the echinocandin micafungin (Barachetti et al., 2009; Giordano et al., 2010; Kano et al., 2008; McLellan et al., 2006; Smith and Hoffman, 2010) .

2.7 Aims

The major aim of this thesis was to characterise aspergillosis of the upper respiratory tract in cats. Specific aims were to:

(i) Describe the clinical syndrome including clinicopathologic findings.
(ii) Determine the aetiological agents of disease.
(iii) Determine the *in vitro* and *in vivo* response to antifungal drugs.
(iv) Evaluate the diagnostic utility of serological assays to detect *Aspergillus*-specific antibodies.

The following steps were taken to achieve these goals:

- Cases of aspergillosis were recruited for retrospective and prospective study from around Australia for characterisation of clinicopathologic findings and *in vivo* response to antifungal drugs (Chapter 3 and Chapter 5).

- For initial investigation of the aetiological agents of disease comparative sequence analyses of the internal transcribed spacer (ITS)1-5.8s-ITS2 and fungal mating tests were performed. (Chapter 3).

- For further investigation of the aetiological agents of disease, morphological studies, growth temperature studies, genotyping of mating genes, and additional comparative sequence analyses of the partial betatubulin and calmodulin genes, and phylogenetic analyses were performed on isolates from clinical cases and on reference strains of *A. fumigatus, A. viridinutans* and *A. udagawae.* To determine the *in vitro* response to antifungal drugs, antifungal drug susceptibility testing was performed (Chapter 4).

- An agar-gel immunodiffusion assay and an Ig-G ELISA to detect *Aspergillus* specific antibodies were evaluated using sera collected from cats with aspergillosis (Chapter 6).

Chapter 3. Characterisation of Clinical Disease and Treatment Outcomes in Cats with Upper Respiratory Tract Aspergillosis.

3.1 Background

Give the paucity of information about FURTA at the time this project was initiated, one of my objectives was to create a data-base of clinical cases of FURTA and a bank of fungal isolates. This was achieved over a three year period from 2006 to 2009. This chapter describes clinical features of the disease including anamnesis, physical findings and clinicopathological findings on haematology, serum biochemistry, feline retrovirus testing results, histopathology and microbiology. The aetiological agents are further characterised using PCR and sequencing of the ITS1-5.8S-ITS2 region of the rDNA cluster as described in Chapter two, and phenotypic data (mating tests). In addition, I explore novel treatment regimens for SOA and establish prognostic differences between *A. fumigatus* and cryptic species infections in cats with URTA.

3.2 Main article

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Sinonasal and sino-orbital aspergillosis in 23 cats: Aetiology, clinicopathological features and treatment outcomes

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ABSTRACT

Aetiology, clinicopathological findings and treatment outcomes were documented in 23 cats (1.5–13 years of age) with sinonasal (SNA, $n = 6$) or sino-orbital (SOA, $n = 17$) aspergillosis. Cases recruited retrospectively and prospectively were included if fungal hyphae were identified on cytological or histological examination and the fungal pathogen was identified by PCR and DNA sequencing (ITS1 or ITS1-5.8S-ITS2 regions, rDNA gene cluster).

Fungal culture was positive in 22/23 cases. In cases of SNA, the fungal pathogen was *Aspergillus fumigatus* ($n = 4$), *Neosartorya fischeri* or *A. lentulus* ($n = 1$) or a non-speciated *Neosartorya* spp. ($n = 1$). In all cases of SOA ($n = 17$), the fungal pathogen was identified as *Neosartorya* spp. Nine cats had brachycephalic conformation. Cats with SNA were more likely to be infected with *A. fumigatus* and had a better prognosis than cats with SOA.

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Introduction

Information about aspergillosis affecting the upper respiratory tract (URT) of cats is restricted to individual case reports and several small case series (Peiffer et al., 1980; Wilkinson et al., 1982; Goodall et al., 1984; Halenda and Reed, 1997; Hamilton et al., 2000; Tomsa et al., 2003; Malik et al., 2004; Whitney et al., 2005; McLellan et al., 2006; Kano et al., 2008; Barachetti et al., 2009; Furrow and Groman, 2009; Karnik et al., 2009; Giordano et al., 2010; Quimby et al., 2010; Smith and Hoffman, 2010). Ten of 25 cats in these reports were Persians or Himalayan Persians, suggesting a possible brachycephalic breed predisposition. Sinonasal aspergillosis (SNA) accounted for approximately half of the reported cases, while the rest had orbital involvement (sino-orbital aspergillosis, SOA). There is limited information on the aetiopathological differences between these two disease presentations, although progression of SNA to SOA has been documented (Hamilton et al., 2000).

The identity of fungal pathogens to species level has been reported in nine affected cats: *Aspergillus fumigatus* ($n = 5$), *A. flavus* ($n = 1$), *A. niger* ($n = 2$) and *A. udagawae* ($n = 1$) (Malik et al., 2004; Whitney et al., 2005; McLellan et al., 2006; Kano et al., 2008; Barachetti et al., 2009; Furrow and Groman, 2009; Smith and Hoffman, 2010; Giordano et al., 2010). However, species identification (*A. udagawae*) was confirmed by molecular studies in only one case; this isolate was initially misidentified as *A. fumigatus* based on phenotypic features (Kano et al., 2008). Members of the *A. fumigatus* complex cannot be identified reliably by phenotypic testing alone (Balajee et al., 2005; Vinh et al., 2009). These findings raise the possibility that pathogens other than *A. fumigatus* could be an underdiagnosed cause of URT aspergillosis in cats.

Treatment of the orbital form of disease is particularly challenging and the prognosis for resolution of infection is generally poor (Hamilton et al., 2000; Kano et al., 2008; Barachetti et al., 2009; Giordano et al., 2010). Few cases have been treated successfully (McLellan et al., 2006; Smith and Hoffman, 2010). The objectives of this study were to document the clinicopathological findings, molecular identity of fungal pathogens and treatment outcomes in cats with URT aspergillosis.

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Materials and methods

Cases and samples

Retrospective cases were identified from the medical records of the University Veterinary Teaching Hospital, Sydney (UVTHS) and Veterinary Pathology Diagnostic Services (VPDS), from January 1998 to December 2006. Cases were recruited prospectively from January 2007 to December 2009 following an Australia-wide call for cases (Barrs et al., 2007). Inclusion criteria were (1) identification of fungal hyphae on cytological or histological examination of tissue biopsies or sinonasal fungal plaques and (2) molecular identification of the isolate from tissue samples (fresh or formalin-fixed paraffin-embedded tissue) and/or culture material. Cats were classified as having SNA or SOA on the basis of absence (SNA) or presence (SOA) of a retrobulbar mass at initial presentation. Clinical data, tissue samples (fresh and/or formalin-fixed paraffin-embedded tissue) and fungal cultures were collected from each case. Data from postmortem examination were included when available.

Clinical data

Signalment, history, clinical signs, haematology, biochemistry, retrovirus serology, latex cryptococcal antigen titres (LCAT), agar gel immunodiffusion (AGID) serology for *Aspergillus* spp. antibodies, microbiology, histopathology, treatment and outcome were recorded. Treatment response was categorised as complete remission or treatment failure; complete remission was defined as resolution of all signs $\geqslant$3 months after cessation of therapy; other outcomes were assigned to the treatment failure group. Cats that could not be assessed for treatment response were censored.

Morphological identification

Samples were cultured at 28 °C and 37 °C on Sabouraud's dextrose agar with added gentamicin and chloramphenicol when bacterial contamination was likely. Where available, isolates identified as *Neosartorya* spp. were retrieved and subcultured on malt extract agar in pairs at 30 °C for 30 days in the dark and examined for cleistothecia (fruiting bodies) at the colony junction. Antifungal susceptibility testing was performed at the Australian Reference Laboratory in Medical Mycology, Adelaide.

Molecular identification

DNA extraction, PCR amplification of the ITS1 region (fresh and formalin-fixed paraffin-embedded tissue) and/or ITS1-5.8S-ITS2 region (culture material) of the rDNA gene cluster was performed from clinical specimens as described previously using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (Chen et al., 2002; Lau et al., 2007). Sequence identity was determined using BLAST against the GenBank[1] and Centraalbureau voor Schimmelcultures (CBS)[2] databases.

Statistical analysis

Statistical analysis was performed using R version 2.6.2. Cats with SNA and SOA were compared with respect to conformation, clinical signs, fungal isolate and response to treatment. Due to the low expected frequencies in some categories, Fisher's Exact Tests were used in preference to χ^2. The fisher.test() function of *R* was used to calculate the *P* value of conditional independence, the conditional Maximum Likelihood Estimate of the odds ratio and the 95% confidence interval of the odds ratio. Significance was ascribed to a *P* value <0.05.

Results

Clinical and clinicopathological findings

Twenty-three cases (4 retrospective, 19 prospective) from New South Wales (n = 10), Queensland (n = 9), Victoria (n = 3) and Western Australia (n = 1) met the inclusion criteria. All cats were neutered (13 female, 10 male). The age range was 1.5–13 years (mean 5.3, median 5 years). There were 12 domestic crossbreds (11 domestic short hair, 1 domestic long hair) and 11 pure breeds comprising one Russian Blue, one Cornish Rex and nine cats with brachycephalic conformation (3 Himalayan Persians, 3 Chinchilla Persians, 2 Ragdolls, 1 Exotic Shorthair) (Table 1).

Six cats had SNA and 17 cats had SOA. Lack of orbital involvement was confirmed by advanced diagnostic imaging (CT; n = 5) or surgical exploration of the affected frontal sinus and ipsilateral orbit (n = 1) in all cases of SNA. In cases of SOA, the presence of a retrobulbar mass was confirmed by advanced imaging (computerised tomography or magnetic resonance imaging; n = 11), at surgery or postmortem examination (n = 4) or by histological confirmation of a fungal granuloma where the retrobulbar mass had perforated into the oral cavity ventrally (n = 2). Four of six cats with SNA and 5/17 cats with SOA were brachycephalic; brachycephalic cats were no more likely to have SNA than non-brachycephalic cats. Five of six SNA and 17/17 SOA cases had a history of sneezing and nasal discharge within the preceding 6 months, whereas 8/17 SOA cases had no signs of sinonasal cavity disease at presentation (Table 2). The only significantly different clinical signs between cats with SNA and SOA were exophthalmos ($P < 0.001$) or presence of a mass or ulcer in the pterygopalatine fossa ($P < 0.05$) (Table 2; Fig. 1).

Retrovirus serology and LCAT were negative in all cats tested (FIV: n = 14; FeLV: n = 11; LCAT: n = 9). *Aspergillus* spp. serology (AGID) was positive in 2/2 cats tested (Table 1). Haematological and serum biochemistry findings for nine cats are presented in Table 3. Five cats with SOA and one with SNA were hyperglobulinaemic; these six cats all had *Neosartorya* spp. infections.

Microbiology

Fungal culture was positive in 22/23 cases. In cases of SNA, the molecular identity of fungal pathogens was *A. fumigatus* (n = 4), *Neosartorya fischeri* or *A. lentulus* (n = 1) and a non-speciated *Neosartorya* spp. (n = 1). Fungal isolates from all 17 cases of SOA were identified as *Neosartorya* spp. by DNA sequencing (Table 1). Fourteen *Neosartorya* spp. were subcultured in pairs. Cleistothecia and ascospores were produced by all isolates from at least one pairing (Fig. 2). No isolates were homothallic. Fourteen of 16 isolates of *A. fumigatus* or *Neosartorya* spp. were resistant to fluconazole (Table 4). Only isolates of *Neosartorya* spp. were resistant to itraconazole (3/13), voriconazole (2/11) and/or posaconazole (1/11). All three isolates of *A. fumigatus* tested were susceptible to voriconazole and posaconazole. No isolates were resistant to amphotericin B.

Histopathology

Inflammatory infiltrates were lymphocytic (n = 2), histiocytic and eosinophilic (n = 1) or neutrophilic (n = 1) in nasal mucosal biopsies available from 4/6 SNA cases. Biopsies were available from 13/17 SOA cases. Granulomatous (n = 1) or plasmacytic and eosinophilic (n = 1) rhinitis were evident in nasal biopsies from two cases. Retrobulbar (n = 11) and nasopharyngeal (n = 2) masses were characterised by necrosis and well-vascularised granulomatous inflammation. Granulomas contained central areas of coagulative and liquefactive necrosis with abundant periodic acid-Schiff-positive fungal hyphae. Surrounding zones of inflammation comprised epithelioid macrophages interspersed with variable numbers of eosinophils, neutrophils, lymphocytes and plasma cells extending into a peripheral zone of fibrosis.

Complete postmortem examinations were performed in six cats with SOA. All had granulomatous mycotic invasion of the nasal cavities and paranasal sinuses, with variable invasion of the submucosal tissue, invasion of paranasal soft-tissues ipsilateral to the affected orbit and lysis of bone (Fig. 3). Inflammatory lesions effaced the adjacent skeletal muscle and bone in some cases.

Two cats that had surgical exenteration of the right orbit subsequently became blind in the left eye. In one case, there was mycotic involvement of the optic chiasm. In the other case, a retrobulbar mass effaced the left optic nerve. Of nine SOA cases in which ocular

[1] http://www.ncbi.nlm.nih.gov/genbank/.
[2] http://www.cbs.knaw.nl/databases/.

Table 1
Case details, molecular identity of fungal pathogens and treatment outcomes in 23 cases of feline upper respiratory tract aspergillosis.

Case	Sex[a]	Age (years)	Breed[a]	Disease[b]	Fungal culture[c]	*Aspergillus* serology[c]	Fungal species (% identity)[d]	Surgery[g]	Number of topical treatments[h]		Systemic antifungals[h]						Treatment (months)	Outcome[i]	Disease free after cessation of therapy (months)
									Clo	Enil	AMB-D	AMB-L	Itra*	Pos*	Vor*	Ter			
1	F	2.6	Persian	SNA	+	ND	*A. fumigatus*[f] (100%)	R	0	0	+	–	+	+	–	+	7	CR	28
2	M	13	DLH	SNA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	ST	0	0	+	+	–	+	–	+	6	CR	24
3	M	5	DSH	SNA	–	ND	*A. lentulus/N. fischeri*[f] (100%)	ST	0	0	+	–	+	–	–	–	7	CR	50
4	F	11	Persian	SNA	+	+	*A. fumigatus*[e] (100%)	–	0	1	+	–	+	+	+	–	9	TF	–
5	F	6.8	Ragdoll	SNA	+	ND	*A. fumigatus*[e](100%)	ST	1†	0	–	–	–	+	–	–	<1	C	–
6	M	7.4	Persian	SNA	+	ND	*A. fumigatus*[e] (99%)	ST	2‡	0	–	–	–	+	–	–	7	CR	7
7	F	5	CR	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e](99%)	–	0	0	+	–	+	+	+	+	16	CR	19
8	F	8	Exotic SH	SOA	+	ND	*N. pseudofischeri*[e,f] (100%)	Ex	0	0	+	–	+	–	–	–	2	C	–
9	F	3.3	Himalayan	SOA	+	ND	*N. aureola/N. udagawae*[e] (99%)	Ex	0	0	–	–	+	+	–	–	1.3	TF	–
10	M	3.6	DSH	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	Ex	0	0	+	–	–	+	+	+	3	TF	–
11	F	2	DSH	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	Ex	0	0	+	–	–	+	–	+	3	TF	–
12	F	2.7	DSH	SOA	+	+	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	Ex	0	0	–	–	+	–	–	–	3	TF	–
13	F	7	DSH	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	Ex	0	0	–	–	–	+	–	–	4.5	C	–
14	M	4	RB	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	Or	0	0	+	–	+	–	–	–	2	TF	–
15	M	2	Himalayan	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	–	0	0	+	–	–	+	–	+	2	TF	–
16	M	2.3	DSH	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	–	0	0	–	–	–	–	–	–	0	NT	–
17	F	4	DSH	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	–	0	0	–	–	–	+	–	–	4.5	TF	–
18	F	5	DSH	SOA	+	ND	*N. fischeri/N. aureola/N. udagawae*[e] (99%)	–	0	0	–	–	–	+	–	+	10	C	–
19	F	8	Himalayan	SOA	+	ND	*N. aureola/N. udagawae*[f] (99%)	–	0	0	+	–	–	+	+	–	2.5	TF	–
20	F	5.9	DSH	SOA	+	ND	*N. aureola*[e] (99%)	–	0	0	+	–	+	+	–	–	8	TF	–
21	M	1.5	DSH	SOA	+	ND	*N. aureola*[e] (99%)	–	0	0	–	–	–	–	–	–	0	NT	–
22	M	8.2	DSH	SOA	+	ND	*N. aureola*[e] (99%)	–	0	0	–	–	–	+	–	–	1	TF	–
23	M	4.7	Ragdoll	SOA	+	ND	*N. aureola*[e] (99%)	–	0	0	–	–	–	+	–	–	6	TF	–

[a] M, male (neutered); F, female (neutered); DLH, domestic longhair; DSH, domestic shorthair; CR, Cornish Rex; Exotic SH, exotic shorthair; RB, Russian blue.
[b] SNA, sinonasal aspergillosis; SOA, sino-orbital aspergillosis.
[c] + Positive; – negative; ND, not done.
[d] % identity with GenBank and Centraalbureau voor Schimmelcultures (CBS) sequences, rounded to nearest whole integer.
[e] ITS1-5.8S-ITS2 PCR from fungal culture.
[f] ITS1 PCR from paraffin-embedded or fresh tissue.
[g] R, ventral rhinotomy; ST, sinus trephination; Ex, exenteration (orbital); Or, orbitotomy plus orbital debridement; –, not treated surgically.
[h] Clo, clotrimazole; Enil, enilconazole; AMB-D, amphotericin B deoxycholate; AMB-L, liposomal amphotericin B; Itra, itraconazole; Pos, posaconazole; Vor, voriconazole; Ter, terbinafine; + treatment prescribed; – treatment not prescribed; * azole drugs were prescribed sequentially not simultaneously; † topical 1% clotrimazole in polyethylene glycol plus 2% clotrimazole cream (Sissener et al., 2006); ‡ topical 1% clotrimazole in polyethylene glycol.
[i] CR, complete remission; TF, treatment failure (euthanased); NT, not treated (euthanased at diagnosis); C, censored.

histology was performed, none had mycotic invasion of the globe, but one cat had anterior uveitis.

Treatment regimes

Treatment was attempted in all six cases of SNA and 15/17 cases of SOA. Two cats with SOA were euthanased when treatment was declined. All 21 treated cats received systemic antifungal therapy. Treatment regimes included amphotericin B plus itraconazole or posaconazole ($n = 5$), amphotericin B plus itraconazole or posaconazole plus terbinafine ($n = 7$) or monotherapy with itraconazole ($n = 2$), posaconazole ($n = 8$) or voriconazole ($n = 4$) (Tables 1 and 5). Some cats received more than one treatment regime. Debridement of fungal plaques within the SNA was performed in 5/6 SNA cases, of which three were also treated with a topical antifungal azole.

Table 2
Clinical findings at presentation in 23 cats with upper respiratory tract aspergillosis.

Clinical findings	SNA[a] ($n = 6$)	SOA[b] ($n = 17$)
Nasal discharge	4	8
Stertor	4	8
Epistaxis	2	0
Temperature ⩾ 39.2 °C	2/4	5/12
Signs of sinonasal cavity disease at presentation	6	9
History of nasal discharge or sneezing within preceding 6 months	5	17
Discharging sinus	2	0
Unilateral exophthalmos	0	16
Bilateral exophthalmos	0	1
Exophthalmos	0	17
Corneal ulcer	0	6
Pterygopalatine fossa mass	0	13
Pterygopalatine fossa ulcer	0	2
Hard palate ulcer	0	4
Paranasal soft tissue swelling	0	6
Pain on opening mouth	0	2
Mandibular lymph node enlargement	2	8/13
Peripheral vestibular disease	0	1

[a] SNA, sinonasal aspergillosis.
[b] SOA, sino-orbital aspergillosis.

Treatment outcomes

Five cases had complete remission (4 SNA, 1 SOA). In the treatment failure group, there were 12 cats (1 SNA, 11 SOA). Four cats were censored (1 SNA, 3 SOA). Cats with SNA were significantly more likely to respond to treatment than cats with SOA ($P < 0.05$). The four SNA cases with complete remission had resolution of clinical signs at follow-up 7, 24, 28 and 50 months after cessation of treatment (Table 1). The SNA case with treatment failure was euthanased 13 months after diagnosis with persistent nasal signs and chronic kidney disease. Complete remission was achieved in one SOA case treated with oral itraconazole and amphotericin B (Table 1, Case 7). However, when treatment was stopped after 8 months, the cat developed recurrent disease and was treated with posaconazole (32 weeks) and terbinafine (16 weeks). The cat remained asymptomatic, with resolution of disease on CT examination, until 19 months after cessation of therapy, when it developed recurrent SOA. Clinical signs persisted despite treatment with posaconazole and liposomal amphotericin B. Clinical signs and CT evidence of infection resolved after treatment with caspofungin, then posaconazole monotherapy, which was ongoing 12 months later.

All SOA cases with treatment failure were euthanased due to progressive disease. Five cats treated with systemic antifungal agents (mean treatment period 2.6 months) and orbital exenteration developed neurological signs, including blindness or reduced vision ($n = 4$), circling and pleurothotonus ($n = 1$), facial muscle fasciculation and stargazing ($n = 1$) or hyperaesthesia ($n = 1$). Of six cats treated with medical therapy alone (mean treatment period 3.9 months), one developed ataxia and paresis.

Of the four cases that were censored, one died during anaesthetic recovery and postmortem examination was declined (Table 1, Case 5), one had resolution of signs but died 1 month after treatment stopped (no postmortem examination performed, Case 13), treatment was ongoing in one (case 18) and one was euthanased with heart failure (hypertrophic cardiomyopathy, Case 8).

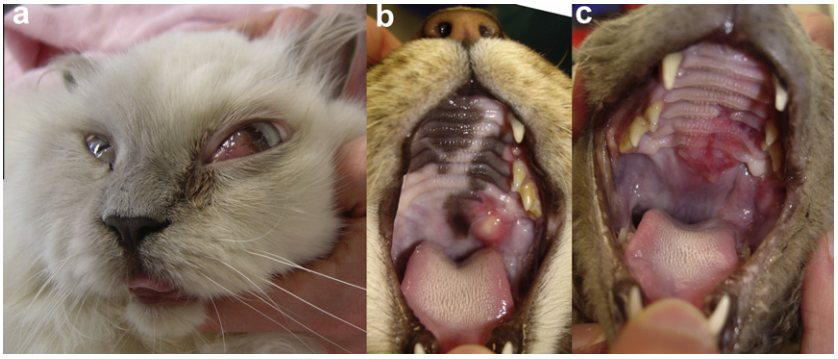

Fig. 1. Exophthalmos (a), mass in the left pterygopalatine fossa (b) and ulceration of the hard palate (c) due to retrobulbar fungal granulomas in cats with sino-orbital aspergillosis.

Table 3
Haematology and serum biochemistry values in nine cats with upper respiratory tract aspergillosis.

Variable	Median (range)	Number with low values	Number with high values	Reference range
Haematocrit (%)	38 (30–48)	0	1	30–45
Total leucocytes ($\times 10^9$/L)	9 (6.5–18.5)	3	2	8–14
Neutrophils ($\times 10^9$/L)	5.2 (2.9–13.7)	1	2	3.8–10.8
Band neutrophils ($\times 10^9$/L)	0	0	0	0–0.4
Monocytes ($\times 10^9$/L)	0.5 (0–1.1)	2	2	0.08–0.6
Lymphocytes ($\times 10^9$/L)	5.3 (0.9–5.0)	1	0	1.6–7
Eosinophils ($\times 10^9$/L)	0.890 (0.1–2.1)	1	1	0.2–1.4
Albumin (g/L)	30 (24–39)	1	0	19–38
Globulin (g/L)	56 (40–106)	0	6	26–51
Urea (mmol/L)	5.5 (4.6–8.1)	0	0	3–10
Creatinine (mmol/L)	119 (100–151)	0	0	90–180
Calcium (mmol/L)	2.5 (2.2–2.8)	0	2	1.75–2.6
Alanine amino transferase (IU/L)	33 (3–64)	0	1	<60
Alkaline phosphatase (IU/L)	20 (13–62)	0	1	<50

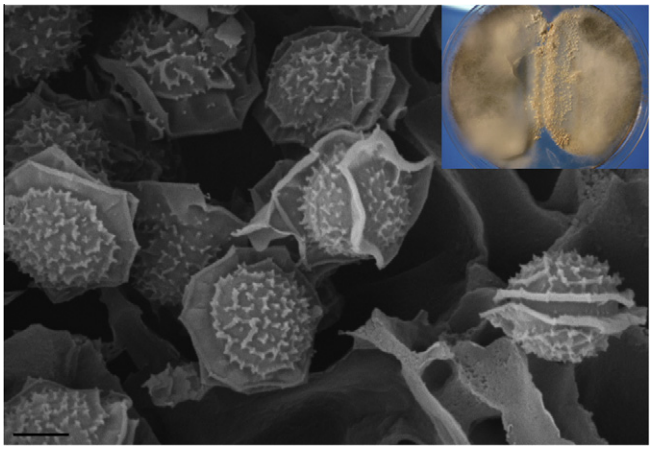

Fig. 2. Cleistothecia (small spherical structures) at the colony junction in paired cultures of *Neosartorya* spp. isolates from two cases (inset). *Neosartorya* spp. ascospores with roughened side walls and two axial crests. Scanning electron micrograph (Zeiss EVO LS15). Scale bar = 2 μm.

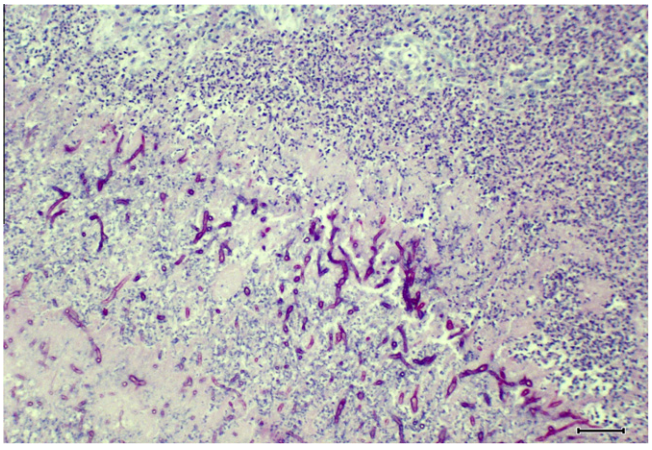

Fig. 3. Periodic acid-Schiff-stained section of frontal sinus epithelium from a cat with invasive sino-orbital aspergillosis. Fungal hyphae are present deep within the sinus epithelium, demonstrating the invasive nature of this mycosis. Scale bar = 60 μm.

Discussion

URT aspergillosis is an emerging infection in cats (Barrs et al., 2007). In our study, all cats were infected with fungi within the complex designated *Aspergillus* section *Fumigati* subgenus *fumigati*, also termed the *A. fumigatus* complex. This complex contains asexual members (anamorph, *Aspergillus* spp.), some of which also have sexual forms (teleomorph, *Neosartorya* spp.). *N. fumigata*, the teleomorph of *A. fumigatus*, has only been induced in vitro after an incubation period of 6 months (O'Gorman et al., 2009). Our ability to induce teleomorphs with relative ease in vitro within 30 days provides additional phenotypic evidence that species other than *A. fumigatus* cause feline SOA and that these fungal pathogens are not strict anamorphs. However, induction of teleomorphs is impractical for routine identification, since clinical isolates may not produce fruiting bodies in the laboratory setting and complementary mating strains are required for heterothallic species (Balajee et al., 2007; Kano et al., 2008).

For comparative purposes, we performed ITS1 and/or ITS1-5.8s-ITS2 PCR and sequencing of archival tissues from the sinonasal cavity of seven dogs with SNA; similar to other reports, the molecular identity of all seven canine mycoses was *A. fumigatus* (Peeters et al., 2005; Windsor et al., 2006). *Neosartorya* spp. has not been identified in URT aspergillosis of dogs; however, in canine SNA where fungal pathogens were identified by phenotypic features alone, it is possible that some cases were caused by members of the *A. fumigatus* complex and mistakenly identified as *A. fumigatus*. There is growing evidence that infections with *Neosartorya* spp. are more common in humans with invasive pulmonary aspergillosis than earlier thought, having been misidentified previously as *A. fumigatus* (Balajee et al., 2005; Vinh et al., 2009; Sugui et al., 2010). A polyphasic taxonomic approach is the gold standard for species identification within the *A. fumigatus* complex. This involves a combination of macro- and micromorphological traits, growth temperature regimes, extrolite profiles and PCR-based detection methods (Balajee et al., 2007; Samson et al., 2007).

The pan-mycotic PCR used in this study can be used to identify a diverse range of fungal genera from clinical specimens, including both filamentous fungi and yeasts (Lau et al., 2007). However, as demonstrated here, the ITS1 and ITS2 regions flanking the 5.8SrDNA may contain insufficient variation to enable identification of some individual species within the *A. fumigatus* complex. After ITS sequence analysis, comparison of partial β-tubulin gene sequences, alone or together with partial rodlet A gene sequences, is recommended for species identification (Balajee et al., 2007; Sugui et al., 2010).

In our study, SOA ($n = 17$) was more frequent than SNA ($n = 6$). Only cats with SNA were infected with *A. fumigatus*, while cats with SOA were infected with other species from the *A. fumigatus* complex. The reason for such a high proportion of SOA cases in this study could in part be due to increased recognition of the disease (Barrs et al., 2007). The ecological niche of species within the *A. fumigatus* complex is the soil and *A. fumigatus* is distributed worldwide (Latge, 1999; Samson et al., 2007). Some *Neosartorya* spp. have a worldwide distribution, whereas others are geographically restricted.

Cases of SOA have been reported in cats from the USA, Italy and Japan (Peiffer et al., 1980; Halenda and Reed, 1997; Hamilton et al., 2000; McLellan et al., 2006; Barachetti et al., 2009; Quimby et al., 2010; Smith and Hoffman, 2010). The molecular identity of the causative agent was confirmed in one case as *A. udagawae* (Kano et al., 2008).

SOA is an invasive mycosis of apparently immunocompetent cats. All cats in this study had URT signs, supporting the sinonasal cavity as the primary site of infection. Furthermore, there was involvement of the sinonasal cavity in all of the cases submitted for postmortem examination. Erosions were found in the orbital lamina, adjacent to the sinonasal cavity, suggesting that extension

Table 4
Results of in vitro antifungal susceptibility tests.

Fungal species	Voriconazole			Posaconazole			Itraconazole			Fluconazole		Amphotericin B	
	S	I	R	S	I	R	S	I	R	S	R	S	I
Aspergillus fumigatus	3	0	0	3	0	0	1	2	0	0	3	2	1
Neosartorya spp.	5	4	2	7	3	1	3	7	3	2	11	10	3

Numbers of isolates susceptible (S), with intermediate/dose-dependent susceptibility (I) or resistant (R).

Table 5
Drugs and dosages used in treatment of 21 cats with upper respiratory tract aspergillosis.

Drug	Number of cats treated	Dose	Dose comments[a]	Adverse effects
Amphotericin B deoxycholate	12	0.5 mg/kg in 350 mL 0.45% NaCl by SC infusion 2–3 times weekly	MCD 9 mg/kg (range 5.5–14 mg/kg)	
Liposomal amphotericin B	2	1–1.5 mg/kg IV every 48 h	MCD 5 mg/kg	
Caspofungin	1	1 mg/kg IV every 24 h	MCD 22 mg/kg	
Voriconazole	4	5–12 mg/kg PO every 24 h	MD 8.3 mg/kg/day. Treatment duration 4–21 days	Anorexia, dilated pupils, hind limb ataxia (3/4 cats)
Itraconazole	9	5–20 mg/kg PO every 24 h	MD 12 mg/kg PO 24-hourly	
Posaconazole	15	2.5–4.5 mg/kg PO every 12 h	MD 3 mg/kg PO 12-hourly	1.1–2× elevations in alanine amino transferase activity (2/10 cats)
Clotrimazole	2	1% topical preparation in polyethylene glycol	Sinonasal instillation (via sinus trephination)	
Enilconazole	1	1% topical preparation	Sinonasal instillation	
Terbinafine	7	12.5–20 mg/kg PO every 12–24 h	15 mg/kg PO 12-hourly	

[a] MCD, mean cumulative dose; MD, mean dose.

of disease occurs via this route. Lysis of the orbital lamina has been identified on CT in cats with SOA and progression from SNA to SOA has been documented (Hamilton et al., 2000; Barachetti et al., 2009).

More than one-third of cats in our study were pure-bred and had a brachycephalic conformation, suggesting that brachycephalic cats are predisposed to URT aspergillosis. Since cats were recruited from around Australia, comparison with local demographic data was not possible. However, data from two Australian states show that there are 2–3 times more domestic cross-bred cats than pure-bred cats and that Burmese cats are the most popular pure-bred cat, comprising up to 25% of the pure-bred population (Lederer et al., 2009; Toribio et al., 2009; NSW Cat Fancy Association kitten registration data 2001–2009, unpublished data). In our study, brachycephalic cats were no more likely to have SNA than non-brachycephalic cats. Furthermore, we identified URT aspergillosis in 13 females and 10 males, which does not support the preponderance of males reported previously (7 females, 14 males).

The basis for an association between brachycephalic conformation and URT aspergillosis in cats is not clear. In humans, decreased sinus aeration and drainage of respiratory secretions secondary to infection, polyps and allergic rhinosinusitis are risk factors for invasive SNA (Siddiqui et al., 2004). Reduced drainage of URT secretions due to brachycephalic conformation could be a risk factor in cats. However, since brachycephalic dogs are under-represented for SNA, it is likely that additional risk factors are present in cats. These could include heritable defects in mucosal immunity, previous viral URT infection and previous antibiotic treatment favouring fungal colonisation (Tomsa et al., 2003).

There is no evidence in our study or in previous reports of an association between retrovirus infection and URT aspergillosis, with only one FeLV positive case reported (Goodall et al., 1984). Hyperglobulinaemia, the most common biochemical abnormality in cats in our study, was documented in four previous cases of SOA (Hamilton et al., 2000; McLellan et al., 2006; Smith and Hoffman, 2010). Chronic antigenic stimulation may explain this hyperglobulinaemia, although electrophoresis was not carried out to determine whether specific immunoglobulin peaks were evident.

The majority of isolates in this study were resistant to fluconazole, which is typical of fungi in the *A. fumigatus* complex (Alcazar-Fuoli et al., 2008). *A. fumigatus* isolates in our study were susceptible to triazole antifungals and amphotericin B, which is also typical for this species (Alcazar-Fuoli et al., 2008). Elevated minimum inhibitory concentrations of voriconazole for *Neosartorya* spp. have been described previously (Balajee et al., 2005).

Presence or absence of orbital involvement in cats with URT aspergillosis has important prognostic significance. Complete remission was significantly more likely in cats with SNA than SOA. Further studies are required to investigate whether the infecting fungal species is a major determinant of treatment outcome. While SOA was only caused by species other than *A. fumigatus*, these cases may be more responsive to treatment if diagnosed before orbital extension. Two out of three SNA cases with complete remission had infections with *Neosartorya* spp. or *N. fischeri*/*A. lentulus*.

Due to the potential for SNA to progress to SOA, systemic antifungal treatments were used in all cases. In all SNA cases with complete remission, gross fungal plaques within the sinonasal cavity were surgically debrided, which could have contributed to treatment success, as reported for canine SNA (Zonderland et al., 2002). Favourable outcomes have resulted from the use of single topical clotrimazole treatment alone ($n = 2$) or in combination with systemic antifungal agents ($n = 1$) in cats with SNA (Tomsa et al., 2003; Furrow and Groman, 2009). Two cats were disease free 2 and 4 years post-infusion. In our study, one case of SNA resolved after a second topical clotrimazole treatment. Multiple topical clotrimazole treatments can improve outcomes in refractory cases of canine SNA (Pomrantz and Johnson, 2010). Whether SNA due to *A. fumigatus* in cats is more likely to respond to topical antifungal therapy than infections due *Neosartorya* spp., which have an increased propensity for invasion, is not yet clear.

Pharmacological data for posaconazole and echinocandins in cats is lacking. Posaconazole was used in our study because it is fungicidal against *Aspergillus* spp. and was reported to cure a feline SOA case that did not respond to itraconazole and amphotericin B (McLellan et al., 2006). Posaconazole was well tolerated, with infrequent mild transient liver enzyme elevations. Use of echinocandins in humans with invasive aspergillosis is restricted to salvage therapy or combination antifungal therapy in refractory disease (Patterson, 2006). In our study, caspofungin was well tolerated and efficacious in the single cat receiving this agent. Kano et al. (2008) documented unsuccessful treatment using micafungin in a cat with SOA.

Adverse effects seen in three cats receiving voriconazole in our study were similar to those reported in five other cats (Quimby et al., 2010; Smith and Hoffman, 2010). The pharmacology of voriconazole has not been studied in cats and caution is urged in the continued use of this drug given the apparent high frequency of serious neurological adverse effects.

Conclusions

Cats with SNA are significantly more likely to be infected with *A. fumigatus* and have a better prognosis than cats with SOA. SOA is

an invasive mycosis in cats and is caused by fungal species within the *Aspergillus* complex other than *A. fumigatus*, such as *Neosartorya* spp.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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3.3 Conclusions

Data from the large cohort described in this publication, combined with previous case reports, establishes important aetiological, epidemiological and diagnostic information about FURTA. Our group first published evidence that cryptic species were systematically implicated in feline URTA in a meeting abstract (Barrs et al., 2007). The publication in this chapter builds on that information, increasing the number of cases studied from seven to twenty three. The next corroborating evidence that cryptic species were implicated in URTA appeared in a case report from Japan describing a case of *A. udagawae* infection in a cat with SOA (Kano et al., 2008). Here, we provide preliminary evidence of an association between anatomic form of FURTA and aetiological agent, with the finding that *A. fumigatus* was only isolated from cases of SNA, and that SOA was only caused by cryptic species. An exciting finding was that 14 of the 17 cryptic species were identified as heterothallic on mating tests. This opened up the possibility that SOA was caused predominantly by a single heterothallic species of *Aspergillus*, and led to the next stage of research described in Chapter four.

My study corroborates the findings from case-reports of FURTA since 1982, that there is no evidence of systemic immune-compromise in the majority of cats with this disease. Unlike disseminated invasive aspergillosis in cats, which is frequently associated with prolonged corticosteroid therapy or concurrent viral immunosuppression from feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV), feline panleukopenia virus or feline infectious peritonitis virus (Bolton GR and TT, 1972; Burk RL et al., 1990; Fox et al., 1978; Köhler H et al., 1978; Ossent, 1987), with the exception of one cat with concurrent FeLV published at a time when FeLV was more prevalent (Goodall et al., 1984), no such associations have been found in cats with FURTA.

Through the cohort described here a predilection for pure-bred cats of brachycephalic conformation was identified. This risk factor for infection raises further questions for research – is disease in these cats due to a conformational abnormality of the skull affecting sinus aeration and drainage? Do these closely related cats have an inherited disorder of innate immunity?

The findings from this study, also establish, that at least in Australia SOA, the more invasive anatomic form of disease, is the most common clinical presentation and that this form has a poor prognosis. We demonstrate that *Aspergillus* isolates can be easily cultured from tissue biopsies from the sinonasal cavity or orbit of infected cats, an important finding that has established culture as the gold standard for diagnosis of FURTA. Further, achieving a definitive diagnosis of this condition should be within the skill set of most veterinary practitioners since specialized fungal culture media or conditions were not required.

The therapeutic approaches adopted for cats with SOA were similar to those used to treat SOA in immunocompetent humans (Panda et al., 2004; Panda et al., 2008; Pushker et al., 2011; Sivak-Callcott et al., 2004). Treatment outcomes were poor despite lack of evidence of systemic immune compromise and aggressive therapeutic protocols combining extensive debridement surgery with the use of modern anti-fungal drugs *viz* posaconazole, voriconazole, caspofungin and liposomal amphotericin B. This finding highlights the need for targeted research in this area to investigate the pharmacokinetics and pharmacodynamics of each of these drugs in the feline host. The adverse effects seen in voriconazole-treated cats in this study have been documented elsewhere (Quimby et al., 2010; Smith and Hoffman, 2010). Cats differ to humans and other animals due to their reduced ability to metabolise drugs by glucuronidation due to substantially reduced hepatic microsomal uridine diphosphate-glucuronosyltransferase (UGT) activity (Court and Greenblatt, 2000). In humans posaconazole metabolism has been shown to be mediated predominantly through phase-2 biotransformation via UGT enzyme pathways (Ghosal et al., 2004). Although posaconazole was clinically well tolerated in the cats treated in my study, the optimal dose has not been established.

Chapter 4. Identification of the aetiological agents of feline aspergillosis and discovery of a novel species – *Aspergillus felis* sp. nov.

4.1 Background

Building on the work described in Chapter three, in this publication I perform further morphologic, molecular and phylogenetic analyses of isolates from cats with SOA at the CBS KNAW Fungal Biodiversity Centre in Utrecht to determine their identity, and in doing so, describe a novel heterothallic species in *Aspergillus* section *Fumigati,* namely *A. felis* (neosartorya-morph).

4.2 Main article

Barrs VR, Tineke M. van Doorn TM, Houbraken J, Kidd SE, Martin P, Pinheiro MD, Richardson R, Varga J, Samson RA. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats and dogs 2013. *PLoS One* 8(6): e64871; doi:10.1371/journal.pone.0064871

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Aspergillus felis sp. nov., an Emerging Agent of Invasive Aspergillosis in Humans, Cats, and Dogs

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Abstract

We describe a novel heterothallic species in *Aspergillus* section *Fumigati*, namely *A. felis* (neosartorya-morph) isolated from three host species with invasive aspergillosis including a human patient with chronic invasive pulmonary aspergillosis, domestic cats with invasive fungal rhinosinusitis and a dog with disseminated invasive aspergillosis. Disease in all host species was often refractory to aggressive antifungal therapeutic regimens. Four other human isolates previously reported as *A. viridinutans* were identified as *A. felis* on comparative sequence analysis of the partial β-tubulin and/or calmodulin genes. *A. felis* is a heterothallic mold with a fully functioning reproductive cycle, as confirmed by mating-type analysis, induction of teleomorphs within 7 to 10 days *in vitro* and ascospore germination. Phenotypic analyses show that *A. felis* can be distinguished from the related species *A. viridinutans* by its ability to grow at 45°C and from *A. fumigatus* by its inability to grow at 50°C. Itraconazole and voriconazole cross-resistance was common *in vitro*.

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Introduction

Aspergillosis, a mycosis caused by infection with fungi belonging to the genus *Aspergillus*, occurs in a diverse range of human and animal hosts. In humans, aspergillosis is diagnosed increasingly due to the introduction of novel immunosuppressive regimens among patients undergoing bone-marrow or solid organs transplants, or treatment for malignancies [1]. Further, the prevalence of fungal infections in mammals is predicted to increase due to global warming leading to expansion of the geographic range of pathogenic fungi [2]. Invasive aspergillosis (IA) in humans occurs predominantly in the sinopulmonary tract of immunocompromised individuals after inhalation of *Aspergillus* spp. conidia. Although *Aspergillus fumigatus* is the most common cause of IA, *A. fumigatus*-like species including *A. lentulus*, *A. udagawae* (*Neosartorya udagawae*), *A. novofumigatus*, *N. pseudofischeri* and *A. viridinutans* are being increasingly identified using molecular techniques [3–9]. These cryptic species have reduced or variable susceptibility to antifungal drugs used for standard therapy of IA including amphotericin B and azoles, which is of concern [5,10].

In domestic cats and dogs, in contrast to humans, fungal rhinosinusitis (FRS) is more commonly reported than invasive pulmonary aspergillosis (IPA) [11–12]. We recently described an emerging clinical syndrome of chronic invasive FRS, also known as sino-orbital aspergillosis (SOA), in apparently immunocompetent cats [13]. Disease is characterized by extension of a sinonasal mycosis into the orbit to form an expansive retrobulbar fungal granuloma with progressive invasion of contiguous anatomic structures including the oral cavity, subcutaneous paranasal tissues and central nervous system. Preliminary investigations suggested that the majority of infections were caused by a heterothallic *A. fumigatus*-like species. Here we identify these isolates using phenotypic, physiologic and phylogenetic analyses as *A. felis* sp. nov. and demonstrate that this species is a cause of IA in cats, dogs and humans.

Materials and Methods

Fungal Strains

Twenty isolates of *A. felis* were available for study including 19 from clinical specimens and 1 isolate from an indoor air sample in Germany (Table 1). Seventeen isolates were from domestic cats (15 with FRS and retrobulbar masses (SOA), 1 with sinonasal cavity infection only (sinonasal aspergillosis, SNA), 1 with a thoracic mass), one isolate was from a dog with disseminated IA and one isolate was from a human with chronic IPA. Three *A. felis* isolates, including the human isolate, had been identified and reported previously as *A. viridinutans* [14,15]. Five additional isolates from cats with FRS or IPA were examined including two isolates of *A.*

Table 1. Isolates from clinical specimens and an indoor air sample included in this study.*

Strain no./Other designation	Species	*MAT1*†	Source	GenBank accession number			
				ITS	*benA*	*CalM*	*MAT1*
DTO 131E3	*A. felis*	1	Cat, RBM, Australia	JX021671	-	-	KC797634
DTO 131E4	*A. felis*	2	Cat, RBM, Australia	JX021673	JX021692	-	KC797622
DTO 131E5	*A. felis*	1	Cat, RBM, Australia	JX021674	JX021693	JX021719	KC797627
CBS 130244	*A. felis*	1	Cat, RBM, Australia	JX021675	JX021694	JX021717	KC797630
DTO 131E9	*A. felis*	1	Cat, RBM, Australia	JX021676	JX021696	-	KC797628
DTO 131F1	*A. felis*	1	Cat, RBM, Australia	JX021677	JX021697	-	KC797629
DTO 131F2	*A. felis*	2	Cat, RBM, Australia	JX021678	JX021698	-	KC797623
DTO 131F3	*A. felis*	2	Cat, RBM, Australia	JX021679	JX021699	-	-
CBS 130245 (T) ‡	*A. felis*	2	Cat, RBM, Australia	JX021685	JX021700	JX021715	KC797620
DTO 131F6	*A. felis*	2	Cat, RBM, Australia	JX021680	JX021702	JX021721	KC797624
CBS 130246‡	*A. felis*	1	Cat, SNC, Australia	JX021681	JX021704	JX021724	KC797631
DTO 131G1	*A. felis*	2	Cat, RBM, Australia	JX021682	JX021705	JX021725	KC797625
CBS 130247‡	*A. felis*	1	Cat, RBM, Australia	JX021683	JX021706	JX021726	KC797632
CBS 130248‡	*A. felis*	2	Cat, RBM, Australia	JX021684	JX021707	JX021727	KC797621
CBS 130249	*A. felis*	2	Dog, VH, Australia	JX021686	JX021711	JX021713	-
CBS 130250	*A. felis*	1§	Cat, RBM, UK	JX021689	JX021712	JX021714	KC797633
MK 246, FRR 5679	*A. felis*	2	Cat, TM, Australia¶	-	AY590129	-	KC797626
MK 284, FRR 5680	*A. felis*	2	Cat, RBM, Australia¶	-	AY590130	-	-
MK 285, FRR 5681	*A. udagawae*	1	Cat, RT, Australia¶	-	AY590133	-	-
DTO 131F5	*A. lentulus*	1	Cat, SNC, Australia	-	-	JX021720	-
DTO 131F7	*A. fumigatus*	-	Cat, SNC, Australia	-	-	JX021722	-
DTO 131E7	*A. fumigatus*	-	Cat, SNC, Australia	-	-	-	-
DTO 131G4	*N. pseudofischeri*	1–2	Cat, SNC, Australia	-	-	JX021716	-
CM 5623	*A.felis*	1§	Human, lung, Portugal**	-	-	KC305167	-
GM 02/39††	*A.felis*	-	Human, sputum/BAL, Spain**	-	HQ127257	-	-
CM 4518††	*A.felis*	-	Human, nail, Spain	-	EU310871	-	-
IFM 54303††	*A.felis*	-	Human, Japan	-	AB248299	AB259973	-
CM 3147††	*A.felis*	-	Human, oropharyngeal exudate, Spain	-	EU310843	-	-
DTO 176F1	*A. felis*	2	Indoor air Stuttgart, Germany	-	-	KC305168	-

*DTO, internal culture collection of CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands; RBM, retrobulbar mass; VH, vitreous humor; TM, thoracic mass; RT, respiratory tract; SNC, sino-nasal cavity; T, type strain; BAL, bronchoalveolar lavage.
†Mating type genotype and phenotype; 1, *MAT1-1*; 2, *MAT1-2*.
‡Isolate included in temperature growth studies.
§Mating genotype only, negative mating test result.
¶Previously reported as *A. viridinutans*-like [14].
**Previously reported as *A. viridinutans* in patients with IA [8,15].
††Sequence data only, sequences sourced from GenBank search for *A. viridinutans*.
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fumigatus, *A. udagawae* (1), *A. lentulus* (1) and *N. pseudofischeri* (1) (Table 1).

Except for isolates MK246, MK284 and MK285, which were obtained from the Commonwealth Scientific and Industrial Research Organisation FRR Culture Collection, North Ryde, NSW, Australia [14], all cases of invasive FRS (SOA), IA and IPA were proven invasive fungal infections based on histopathologic and/or cytopathologic detection of hyphae in needle aspiration or biopsy specimens with evidence of associated damage (Figure 1) [16]. For phylogenetic analyses, a PubMed search for *A. viridinutans* isolates from human clinical specimens deposited in GenBank with accession numbers for the internal transcribe spacer (ITS) regions, partial β-tubulin (*benA*) and/or calmodulin (*calM*) sequences was carried out. The sequences of four isolates previously identified as *A. viridinutans* (GM 02/39, CM 4518, IFM 54303, CM 3147) were identical to *A. felis* sp. nov. (Table 1). Reference strains included in the study are listed in Table S1.

Clinical data

Cats with invasive FRS were presented by their owners for veterinary investigation of unilateral exophthalmos caused by a retrobulbar fungal granuloma. All had nasal discharge at

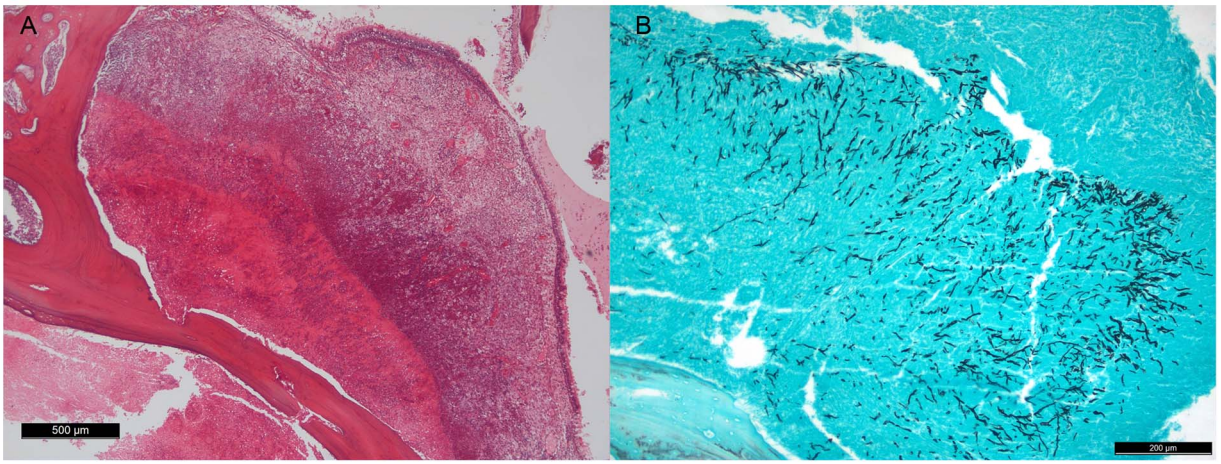

Figure 1. Tissue invasion by fungal hyphae in a cat with SOA. Hematoxin & Eosin- (A) and Grocott- (B) stained section of nasal mucosa and turbinates demonstrating granulomatous rhinitis (A) and submucosal invasion by septate branching fungal hyphae (B).
doi:10.1371/journal.pone.0064871.g001

presentation or historical evidence of sneezing or nasal discharge within the previous 6 months. The cats had no significant intercurrent diseases and were considered to be systemically immunocompetent [13]. The dog with disseminated IA presented with panuveitis, spinal pain, cardiac murmur and fever subsequent to immunosuppressive therapy with cyclosporine and prednisolone for immune-mediated hemolytic anaemia. The human patient with chronic IPA was a 56 year old man with type II diabetes mellitus receiving immunosuppressive therapy (prednisolone, etarnecept and methotrexate) for rheumatoid arthritis. Infection was protracted over 18 months and extended from a solitary lung nodule across anatomic planes to involve cervical lymph nodes and pleural space [15].

Morphological characterization

Isolates were grown for 7 days as 3-point inoculations on Czapek agar, Czapek yeast autolysate agar (CYA), malt extract agar (MEA) and oatmeal agar (OA) at 25°C and 37°C. Media were prepared as described by Samson et al [17]. To determine *MAT1-1* and *MAT1-2* phenotypes, mating tests for teleomorph induction were performed by crossing a selection of isolates on MEA and OA in all possible combinations of opposite mating type and incubating at 30°C in the dark. Additional mating tests were performed using one isolate with confirmed phenotype and genotype of *MAT1-1* (CBS 130246) and of *MAT1-2* (CBS 130245) (Table 1). Cleistothecia produced from each positive mating were crushed and examined microscopically for the presence of ascospores. To determine ascospore viability and heat resistance 4-week old cleistothecia from two paired matings (CBS 130245× DTO 131E9, DTO 131E9× DTO131F3) were ruptured, suspended in 0.05% Tween 80 and heated to 70°C for 60 min as described previously [18]. Aliquots of ascospore suspension (200 µL) were cultured on 5% MEA, incubated at 28°C and examined by light microscopy after 18 h incubation for spore germination (Olympus BH2). Isolates were also mated with type strains of *A. viridinutans* (CBS 127.56), *A. fumigatus* (CBS 133.61) and *A. udagawae* (CBS 114217, CBS 114218).

For radial growth determination, MEA was inoculated with 3 µL of conidial suspension (3×10^7 conidia/mL) in the centre of the plate and colony diameters were measured after 7 days incubation at temperatures ranging from 6°C in increasing increments of 3°C to 33°C, and at 36°C, 40°C, 45°C and 50°C. Morphologic features were studied by light microscopy (Olympus BH2 and Zeiss Asioskop 2 Plus) and by scanning electron microscopy (Zeiss EVO LS15). Temperature growth studies were performed using two replicates each of type strains of *A. viridinutans*, *A. fumigatus* and clinical isolates CBS 130245, CBS 130246, CBS 130247 and CBS 130248 (Table 1 & Table S1).

Phylogeny and Molecular Identification

Isolates were grown on MEA for 7 days at 37°C and genomic DNA was extracted using the Ultraclean microbial DNA isolation kit (MoBio, Solana Beach, CA) according to the manufacturer's instructions. Amplification of the ITS regions, including ITS-1, ITS-2 and the 5.8S rDNA gene (primers ITS1 and ITS4), and parts of the β-tubulin (*benA*) (primers Bt2a and Bt2b) and calmodulin (*calM*) gene (primers cmd5 and cmd6) was performed as described previously [19–21]. Sequencing reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction kit and carried out for both strands. Sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NH), equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Sequence alignments were performed using ClustalW software incorporated in MEGA version 5 [22]. Alignment positions with gaps or missing data were excluded, and all characters were unordered and of equal weight. The maximum parsimony (MP) tree was obtained using the Close-Neighbour-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree was drawn to scale with branch lengths calculated using the average pathway method. To assess the robustness of the topology, 500 bootstrap replicates were run by MP and tree length, and consistency index (CI) and retention index (RI) were calculated. *Aspergillus clavatus* (CBS 513.65) was the out-group in the trees based on *benA* and *calM* and *N. pseudofischeri* (CBS 208.92) was the out-group in the tree based on ITS sequence data. Unique ITS, *benA* and *calM* sequences were deposited in the GenBank nucleotide sequence database under accession numbers: JX021671- JX021727 and KC305167-KC305168 (Table 1).

Degenerate primers were constructed to amplify the alpha domain-encoding sequence from the *MAT1-1* gene family and the high mobility group (HMG) domain-encoding sequence from

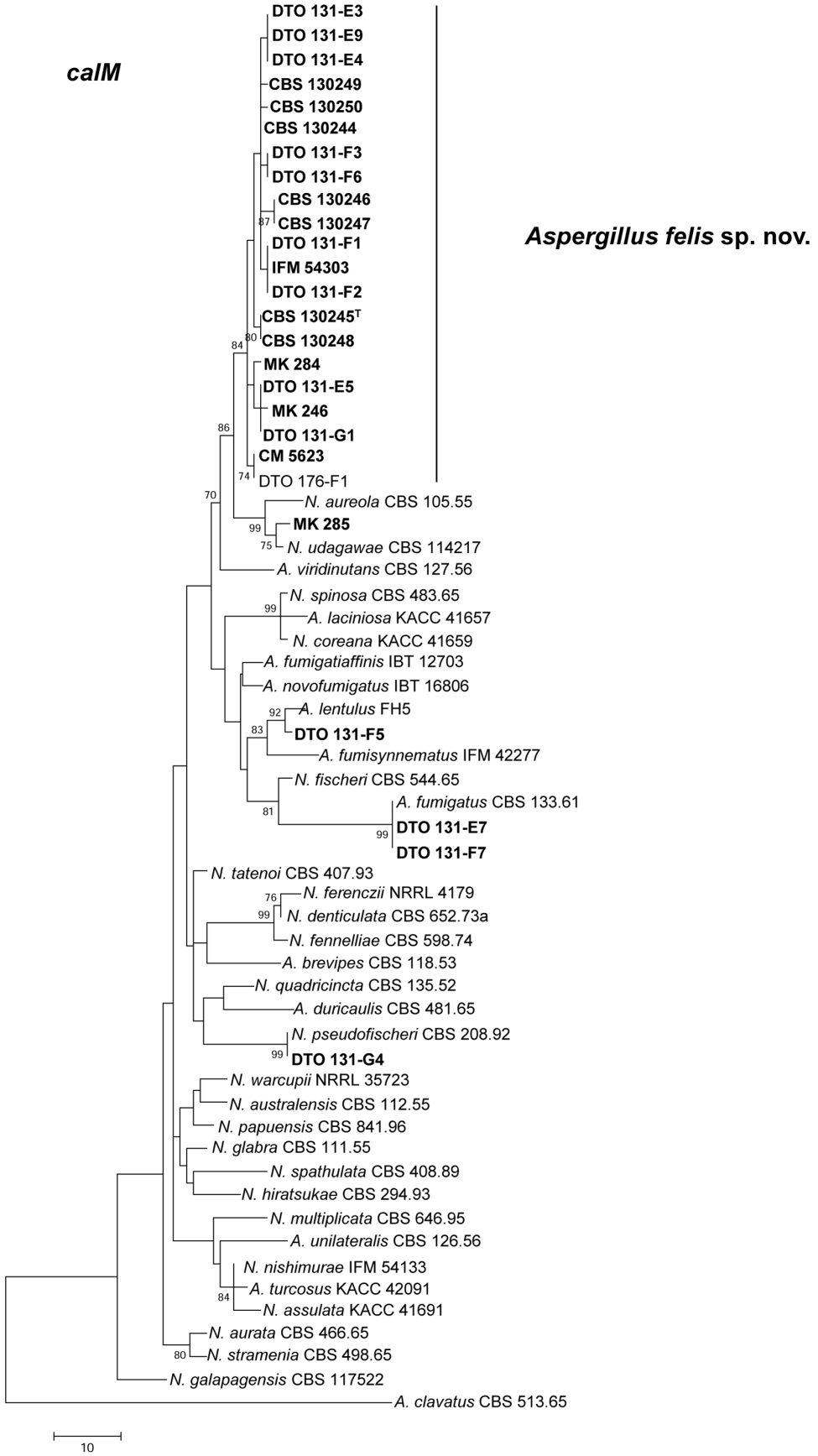
calM
DTO 131-E3
DTO 131-E9
DTO 131-E4
CBS 130249
CBS 130250
CBS 130244
DTO 131-F3
DTO 131-F6
CBS 130246
CBS 130247
DTO 131-F1
IFM 54303
DTO 131-F2
CBS 130245T
CBS 130248
MK 284
DTO 131-E5
MK 246
DTO 131-G1
CM 5623
DTO 176-F1
Aspergillus felis sp. nov.
N. aureola CBS 105.55
MK 285
N. udagawae CBS 114217
A. viridinutans CBS 127.56
N. spinosa CBS 483.65
A. laciniosa KACC 41657
N. coreana KACC 41659
A. fumigatiaffinis IBT 12703
A. novofumigatus IBT 16806
A. lentulus FH5
DTO 131-F5
A. fumisynnematus IFM 42277
N. fischeri CBS 544.65
A. fumigatus CBS 133.61
DTO 131-E7
DTO 131-F7
N. tatenoi CBS 407.93
N. ferenczii NRRL 4179
N. denticulata CBS 652.73a
N. fennelliae CBS 598.74
A. brevipes CBS 118.53
N. quadricincta CBS 135.52
A. duricaulis CBS 481.65
N. pseudofischeri CBS 208.92
DTO 131-G4
N. warcupii NRRL 35723
N. australensis CBS 112.55
N. papuensis CBS 841.96
N. glabra CBS 111.55
N. spathulata CBS 408.89
N. hiratsukae CBS 294.93
N. multiplicata CBS 646.95
A. unilateralis CBS 126.56
N. nishimurae IFM 54133
A. turcosus KACC 42091
N. assulata KACC 41691
N. aurata CBS 466.65
N. stramenia CBS 498.65
N. galapagensis CBS 117522
A. clavatus CBS 513.65
10

Figure 2. Partial calmodulin gene (*calM*) maximum parsimony (MP) tree. Phylogenetic analysis for *A. felis* sp. nov isolates and closely related species as conducted in MEGA5 [22] showing best scoring MP tree constructed using the close-neighbor-interchange algorithm [40]. Bootstrap percentages of the MP analysis are presented at the nodes for values >70%. Trees are drawn to scale, with branch lengths calculated using the average pathway method, expressed in units of the number of changes over the whole sequence. Isolates from clinical specimens used in this study are in bold. Isolate DTO 176-F1 was from an indoor air sample in Germany.
doi:10.1371/journal.pone.0064871.g002

MAT1-2. *MAT1-1* primers (AFM1_F65655 (5′- CCT YGA CGM GAT GGG ITG G –3′) and MAT1_R6215 (5′- TG TCA AAG ART CCA AAA GGA GG –3′) were designed by identifying regions of conserved sequences in *A. clavatus*, *Neosartorya fischeri* and *A. fumigatus*. *MAT1-2* primers (MAT2_F6086 (5′- TCG ACA AGA TCA AAW CYC GTC –3) and MAT2_R6580 (5′- CTT YTT GAR CTC TTC YGC TAG G –3′) were designed by identifying regions of conserved sequence in *A. nidulans*, *N. fischeri* and *A. fumigatus*. PCR reactions were set up as described previously [23]. PCR cycle parameters were denaturation at 95°C for 5 min, then 30 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. *MAT1-1* and *MAT1-2* sequences were deposited in the GenBank nucleotide sequence database under accession numbers KC797620-KC797634 (Table 1).

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 ONE* 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/MycoTaxo.aspx?Link = T&Rec = . The online version of this work is archived and available from the following digital repositories: PubMed Central, LOCKSS.

Antifungal Susceptibility Testing

Susceptibilities of 13 *A. felis* isolates to amphotericin B, itraconazole, posaconazole, voriconazole, fluconazole, 5-flucytosine, caspofungin, anidulafungin and micafungin were assessed using the Sensititre YeastOne YO10 microdilution trays (Trek Diagnostic Systems, Thermo Fisher Scientific, Australia), which have been demonstrated to yield comparable results to the CLSI M38-A standards for molds [24]. Susceptibility to terbinafine (Novartis Pharmaceuticals Corporation, Sydney, Australia) was assessed according to the CLSI M38-A2 standard [25]. Endpoints for all drugs were determined after 48 h incubation at 35°C. For amphotericin B, itraconazole, posaconazole, voriconazole, fluconazole and 5-flucytosine, endpoints were read as the lowest concentration with a complete color change, indicating total inhibition of fungal growth (minimum inhibitory concentration, MIC).The echinocandin endpoints were read as the lowest concentration revealing a partial color change, which also corresponded to the lowest concentration of drug leading to the growth of small compact hyphal forms as compared to the hyphal growth observed in the drug free control (minimum effective concentration, MEC). The terbinafine endpoint was read as the first dilution with 100% growth inhibition (MIC). *Aspergillus flavus* (ATCC 204304) and *Candida parapsilosis* (ATCC 22019) isolates were used as quality control strains.

Results

Sequence-based analyses

The *calM* data set consisted of 487 characters, including 171 parsimony informative sites. MP analysis resulted in 792 equally parsimonious trees (tree length 350 steps, CI 0.547297, RI 0.778512) (Figure 2). Of the aligned *benA* sequences, a region with 394 positions, including 63 parsimony informative characters, was selected for analysis. MP analysis resulted in 241 equally parsimonious trees (length 447, CI 0.583113, RI 0.802253) (Figure 3). The ITS data set consisted of 499 characters, including 34 parsimony informative sites. MP analysis resulted in 377 equally parsimonious trees (length 34, CI 0.800000, RI 0.947917) (Figure S1).

The tree topology inferred from *calM* grouped 17 feline isolates, the canine isolate (CBS 130249), 2 human isolates (CM 5623, IFM 54303), and the air sample (DTO 176) in a clade with 84% bootstrap support that had a most recent ancestor in common with *N. aureola* and *A. udagawae* (86% bootstrap support). *A. viridinutans* was positioned basal to these three species. Five other isolates from cats with FRS were identified as *A. fumigatus* (DTO 131-E7, DTO 131-F7), *A. udagawae* (MK 285), *A. lentulus* (DTO 131-F5) and *N. pseudofischeri* (DTO 131-G4) (Table 1, Figure 2). Analysis of *benA* showed a similar grouping as observed in the MP analysis of *calM*. In addition, three other sequences deposited in GenBank and originating from human clinical specimens (GM 02/39, CM 4518, CM 3147) belonged to the major clade comprising most clinical specimens, with high bootstrap support (95%). *A. viridinutans* was basal to this clade, but this relationship was lacking statistical support (Figure 3). On MP analysis based on ITS sequences 17 feline isolates, the canine isolate and the human isolate CM 5623 clustered together in a monophyletic group (Figure S1). All isolates belonging to this group shared identical ITS sequences. Based on the molecular data, and the phenotypic and physiological data presented below, we decided to name the strains in this clade *Aspergillus felis* sp. nov.

Mating-type analysis

Crosses with opposite mating partners on MEA and OA resulted in cleistothecia and ascospores within 7 to 10 days for all *A. felis* (neosartorya-morph) isolates except for CBS 130250 and CM 5623 (Table 1, Figure 4). Cleistothecia formed in small clusters mainly in the barrage zone and to a lesser extent within adjacent mycelium (Figure 4). Ascospores germinated on MEA after exposure to heat and gave rise to characteristic *A. felis* colonies. The PCR-based mating-type assay successfully amplified the fragments of the alpha- or HMG-domain genes in all *A. felis* isolates. The *MAT* genotype successfully matched the *MAT* phenotype for all isolates with positive mating tests. In addition, the PCR amplicons were sequenced (GenBank KC797620-KC797634) and a homology search on GenBank confirmed their designation to *MAT1-1* or *MAT1-2*. Mating tests with *A. udagawae*, *A. viridinutans* and *A. fumigatus* were negative. Immature cleistothe-

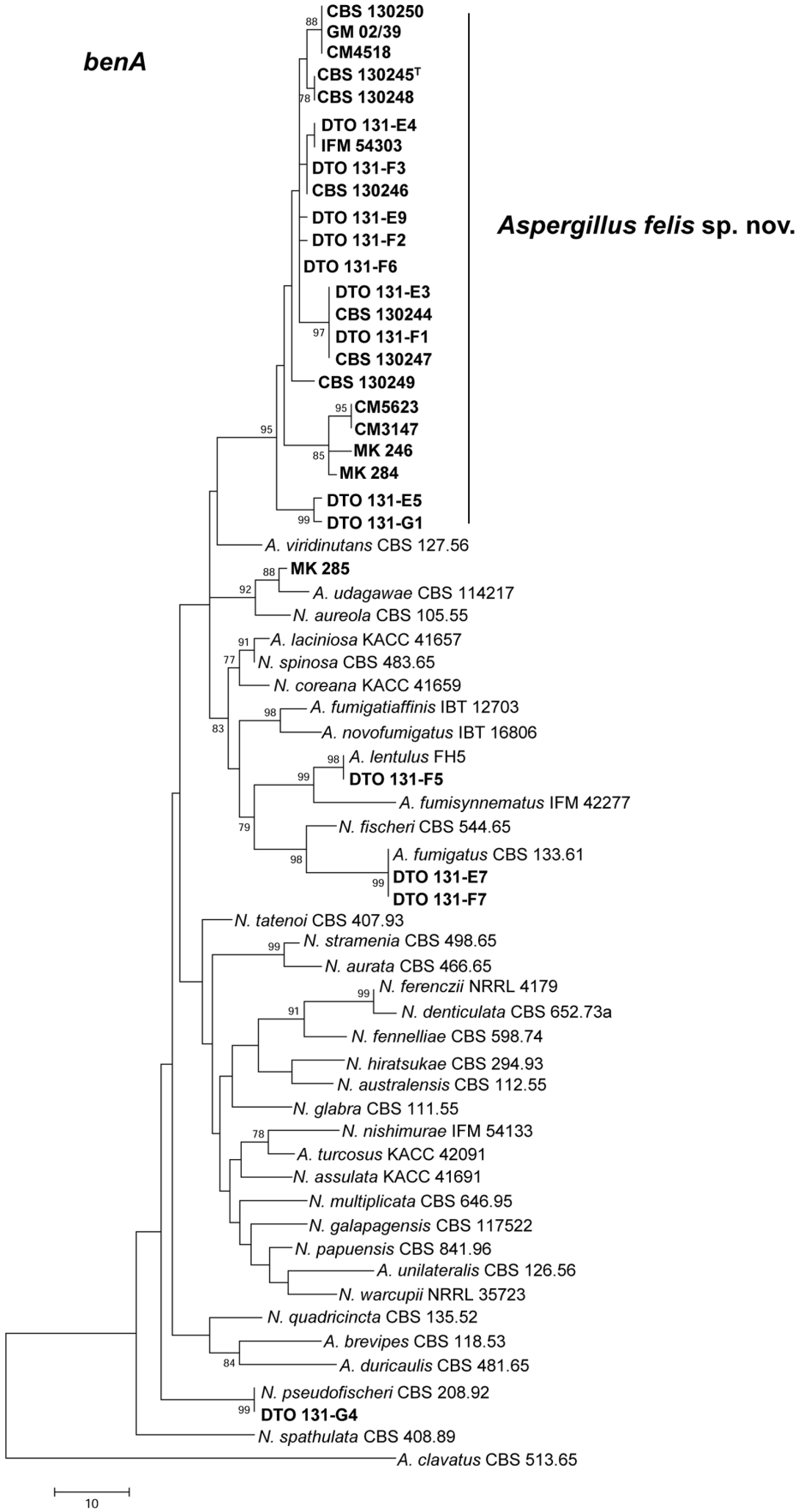
benA
CBS 130250
GM 02/39
CM4518
CBS 130245T
CBS 130248
DTO 131-E4
IFM 54303
DTO 131-F3
CBS 130246
DTO 131-E9
DTO 131-F2
DTO 131-F6
DTO 131-E3
CBS 130244
DTO 131-F1
CBS 130247
CBS 130249
CM5623
CM3147
MK 246
MK 284
DTO 131-E5
DTO 131-G1
Aspergillus felis sp. nov.
A. viridinutans CBS 127.56
MK 285
A. udagawae CBS 114217
N. aureola CBS 105.55
A. laciniosa KACC 41657
N. spinosa CBS 483.65
N. coreana KACC 41659
A. fumigatiaffinis IBT 12703
A. novofumigatus IBT 16806
A. lentulus FH5
DTO 131-F5
A. fumisynnematus IFM 42277
N. fischeri CBS 544.65
A. fumigatus CBS 133.61
DTO 131-E7
DTO 131-F7
N. tatenoi CBS 407.93
N. stramenia CBS 498.65
N. aurata CBS 466.65
N. ferenczii NRRL 4179
N. denticulata CBS 652.73a
N. fennelliae CBS 598.74
N. hiratsukae CBS 294.93
N. australensis CBS 112.55
N. glabra CBS 111.55
N. nishimurae IFM 54133
A. turcosus KACC 42091
N. assulata KACC 41691
N. multiplicata CBS 646.95
N. galapagensis CBS 117522
N. papuensis CBS 841.96
A. unilateralis CBS 126.56
N. warcupii NRRL 35723
N. quadricincta CBS 135.52
A. brevipes CBS 118.53
A. duricaulis CBS 481.65
N. pseudofischeri CBS 208.92
DTO 131-G4
N. spathulata CBS 408.89
A. clavatus CBS 513.65
10

Figure 3. Partial β-tubulin gene (*benA*) maximum parsimony (MP) tree. Phylogenetic analysis for *A. felis* sp. nov isolates and closely related species as conducted in MEGA5 [22] showing best scoring MP tree constructed using the close-neighbor-interchange algorithm [40]. Bootstrap percentages of the MP analysis are presented at the nodes for values >70%. Trees are drawn to scale, with branch lengths calculated using the average pathway method, expressed in units of the number of changes over the whole sequence. Isolates from clinical specimens used in this study are in bold.
doi:10.1371/journal.pone.0064871.g003

cia containing no ascospores were observed along colony junctions for matings of *A. felis* isolates with *A. viridinutans* and *A. fumigatus*.

Morphological and physiological characterization

Phylogenetic analysis showed that *A. felis* is closely related to *N. aureola* and *A. udagawae*. The ascospore morphology supports this

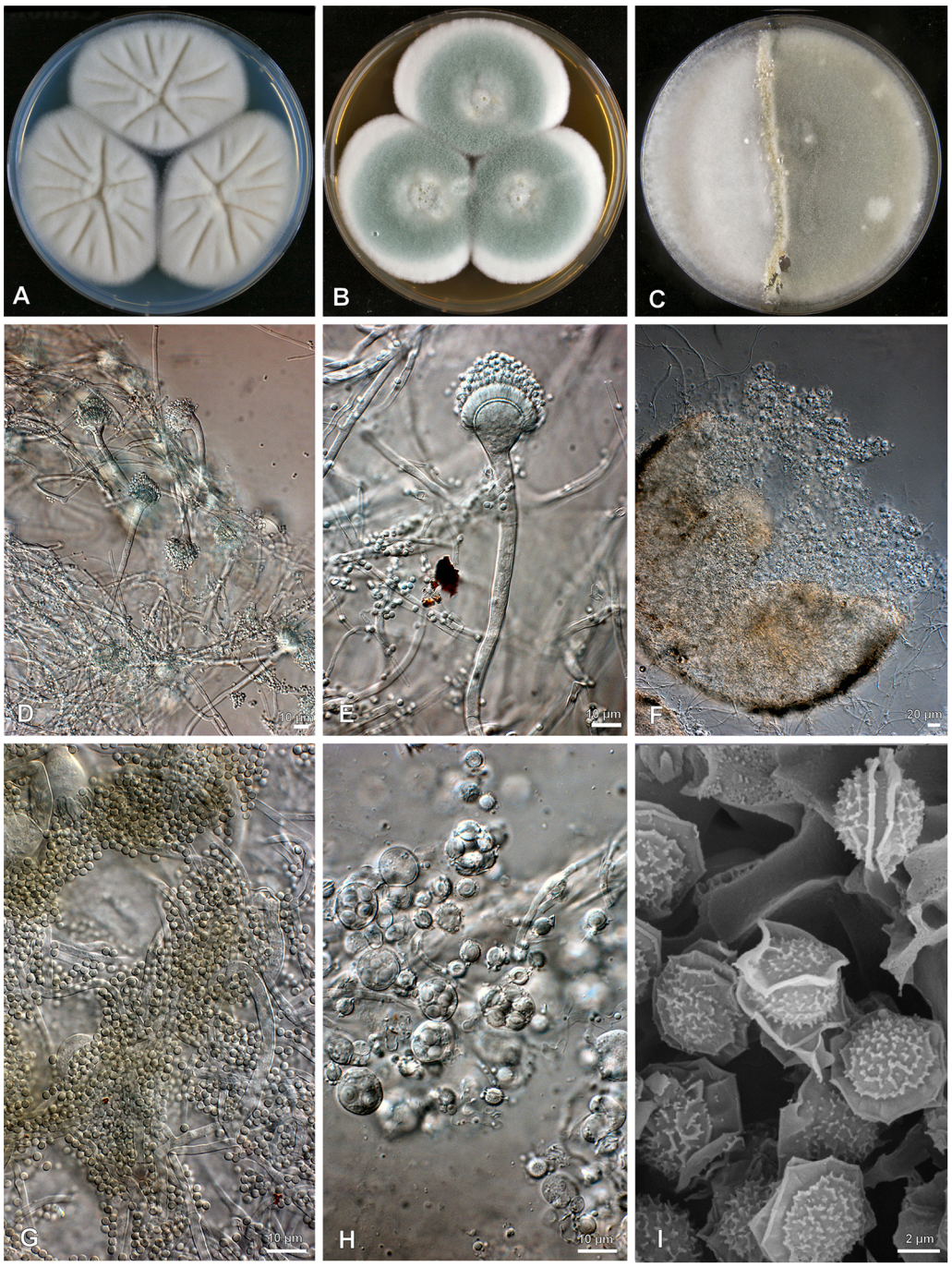

Figure 4. *Aspergillus felis*. Colonies growing 7 days at 25°C on CYA (A) and MEA (B); Crossing of CBS 130245 and 130246 at 30°C (C); Conidiophores and conidia (D, E and G); Cleistothecium (F); Ascospores (H-I).
doi:10.1371/journal.pone.0064871.g004

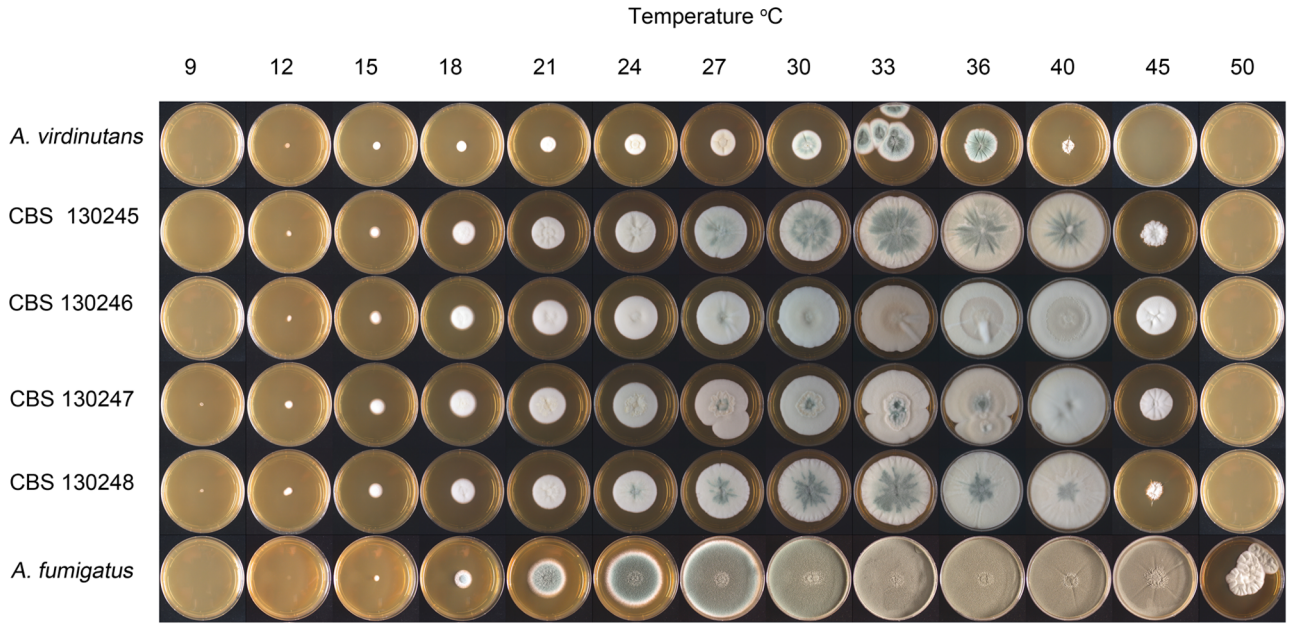

Figure 5. Radial growth determination at temperatures ranging from 9°C to 50°C. Type strains of *A. viridinutans* (CBS 127.56) and *A. fumigatus* (CBS 133.61) and 4 isolates of *A. felis* (CBS 130245, CBS 130246, CBS 130247, CBS 130248).
doi:10.1371/journal.pone.0064871.g005

phylogenetic relationship since *A. felis*, *N. aureola* and *A. udagawae* all produce lenticular ascospores with two prominent equatorial crests and an echinulate convex surface (Figure 4) [26]. However, *A. felis* can be distinguished from *N. aureola* by its heterothallic reproduction mode and from *A. udagawae* by its ability to grow at 45°C (Figure 5), since the maximal growth temperature for *A. udagawae* is 42°C [5]. Also *A. felis* has ascospores which are 5.0–7.0×3.5–5.0 µm compared to 5–5.5×4–5 µm in *A. udagawae* [26] (Figure 4). *A. viridinutans* is phylogenetically and phenotypically related to *A. felis* and both species share the production of "nodding" conidial heads and weakly sporulating colonies (Figure 4), but can be differentiated by maximum growth temperatures. *A. felis* is able to grow at 45°C, while in our study *A. viridinutans* exhibited no growth at 45°C (Figure 5). *A. fumigatus* is phenotypically similar but is able to grow at 50°C, while none of the tested *A. felis* grew at this temperature (Figure 5).

Species description of *A. felis*

Aspergillus felis Barrs, van Doorn, Varga & Samson, **sp. nov.** [urn:lsid:indexfungorum.org:names: Mycobank MB 560382] (Figure 4).

Etymology. named after the first host in which clinical disease was described. *Felis* is a genus of cats in the family Felidae.

Diagnosis. *Aspergillus felis* (neosartorya-morph) is phenotypically similar to *A. viridinutans*, but differs by its ability to grow at 45°C. This species is phylogenetically related to *N. aureola* and *A. udagawae* and differs to *N. aureola* in having a heterothallic reproduction mode [26–27].

Typus: ex. retrobulbar mass in domestic short-haired cat, Australia (CBS H-21125-holotypus, culture ex-type: CBS 130245).

Description. Colonies grow rapidly on CYA agar attaining a diameter of 5.0 to 5.5 cm in 7 days at 25°C and on MEA reach 5.5 cm in diameter in 7 days at 25°C (Figure 4, panels A and B). On CYA the colony texture is mostly floccose; colonies are usually

Table 2. Antifungal susceptibility results for 13 *A. felis* isolates from clinical specimens from cats.*

Drug	MIC/MEC (µg/mL) Distribution Among Tested Isolates										GM
	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	
AMB†						1	11	1			0.50
ITZ†			1	2	2	4	1	3			0.22
VCZ†						1	3	1	5	3	1.38
POS†			4	3	2	1	2	1			0.10
TB†						13					0.25
CSP‡	1		7	4					1		0.05
ANF‡		13									0.015
MCF‡	11	2									0.009

*MIC, minimum inhibitory concentration (†); MEC, minimum effective concentration (‡), GM, geometric mean µg/mL; AMB, amphotericin-B; ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; TB, terbinafine; CSP, caspofungin; ANF, anidulafungin; MCF, micafungin.
doi:10.1371/journal.pone.0064871.t002

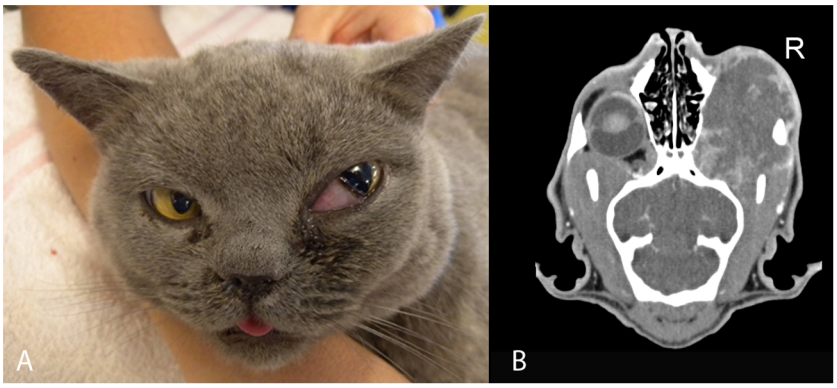

Figure 6. Cat with sino-orbital aspergillosis (invasive fungal rhinosinusitis) caused by A. felis with exophthalmia and prolapse of the nictitating membrane (third eyelid) associated with a retrobulbar fungal granuloma (A). Coronal CT scan soft-tissue post-contrast view showing retrobulbar fungal granuloma occupying the inferior aspect of the orbit with involvement of the adjacent paranasal subcutaneous tissues (B).
doi:10.1371/journal.pone.0064871.g006

white and often sporulate poorly. On MEA colonies are more or less velvety with abundant greenish sporulation occurring after 5 to 7 days. In reverse, colonies are cream to light green. Conidiophores are uniseriate with greenish stipes and subclavate, "nodding" heads (Figure 4, panels D and E). Vesicles are subclavate with a diameter of 15–16.5 µm. Conidia are green, globose to subglobose, finely roughened and 1.5–2.5 µm in dimensions. Cleistothecia are white to creamish, 100–230 µm. Asci are globose, 8-spored, 12–16 µm in diameter (Figure 4, panel H). Ascospores are lenticular with two prominent equatorial crests and with short echinulate convex surfaces 5.0–7.0×3.5–5.0 µm (Figure 4, panel I).

Occurrence. This species had been found in cats with chronic invasive FRS and retrobulbar masses (SOA), IPA or with sinonasal cavity infection only (sinonasal aspergillosis, SNA), in a dog with disseminated IA, in a human with chronic IPA and in an indoor air sample in Germany.

Barcode. GenBank JX021685 (ITS). This species can be identified with ITS, β-tubulin and/or calmodulin sequences.

Taxonomy. In July 2011, the dual nomenclature system was abandoned and replaced with single name nomenclature [28]. We followed the recommendations of the International Commission on *Penicillium* and *Aspergillus* here and describe this species in *Aspergillus*, even though it produces a teleomorphic state. Using the old nomenclature rules, this species would have been included in the genus *Neosartorya*. In accordance with the Amsterdam declaration on fungal nomenclature we refer to the newly discovered teleomorph by an informal cross reference name in lower case Roman type *Aspergillus felis* (neosartorya-morph) [29].

Antifungal susceptibilities

There was no observed activity of fluconazole or 5-flucytosine against *A. felis*. The distribution of MIC/MECs of other antifungals among those isolates that could be tested is shown in Table 2. A bimodal MIC distribution was observed for the triazoles against *A. felis*, with high MICs to at least one of the triazoles observed in four isolates including two isolates that exhibited cross-resistance to ITZ/VCZ and ITZ/VCZ/POS, respectively. Another *A. felis* isolate had a high MEC of caspofungin (2 µg/mL) but MECs of the other echinocandins were comparable to the other isolates tested within this species.

Discussion

Considering the morphological, biological and phylogenetic species concepts, we present evidence for a novel species within *Aspergillus* section *Fumigati*, *A. felis* sp. nov [30]. The *BenA* and *CalM* phylogenies indicate genetic isolation of *A. felis*. We also established reproductive isolation; *A. felis* is a heterothallic fungus with a fully functioning reproductive cycle as determined by teleomorph induction and detection of *MAT1-1* and *MAT1-2* genes by PCR in isolates of corresponding phenotype. Ascospores were demonstrated to be both viable and heat resistant. The heterothallic mode of reproduction for *A. felis* should be confirmed further by the establishment of meiotic recombination in ascospore progeny as was described by O'Gorman and Dyer in their parallel discovery of a functional heterothallic sexual cycle in *A. fumigatus* [18]. No teleomorph has as yet been isolated *in vitro* for *A. viridinutans* although results of mating-type genotype determination in our study indicate it is a heterothallic fungus. Cryptic sexual states are being increasingly identified in mitosporic fungi [31]. For heterothallic fungi a strong correlation has been established between biological species based on mating tests and phylogenetic species based on multilocus sequence typing [32]. Differences in maximum growth temperature were also identified between *A. felis* and the type strain of *A. viridinutans*. *A. felis* is a thermotolerant fungus, as defined by a maximum growth temperature of ≥45°C and a minimum growth temperature of <20°C. The maximal growth temperature of both *A. viridinutans* and *A. udagawae* has been reported as 42°C [5,33].

Species within *Aspergillus* section *Fumigati* cannot be reliably identified on the basis of morphologic criteria alone [4]. In contrast to other *Aspergillus* species, *A. felis* can be reliably identified with ITS sequences only. Molecular identification of *A. fumigatus*-like molds in human patients with IA has important clinical relevance since clinical disease characteristics vary with infecting species and MICs of antifungal drugs, especially triazoles, are often high [5–7,10,34]. This is exemplified in a study of 86 isolates from patients with IA identified as *A. fumigatus* on phenotypic features. When the same isolates were later identified by sequencing the partial *benA* and rodlet A genes, 12 (14%) were identified as *A. udagawae* [5]. In patients with *A. udagawae* infection, the median duration of illness was 7 times longer than in patients with confirmed *A. fumigatus* infection, disease was refractory to standard therapy and MICs of various antifungals of *A. udagawae* isolates were higher than those for *A. fumigatus* [35]. Similarly, a distinctive form of IA characterized by chronicity, propensity to spread across

anatomical planes and reduced antifungal susceptibility was attributed to infection with *A. viridinutans* based on comparative partial *benA* and rodlet A gene sequence analyses [15]. We re-examined this isolate (CM 5623) and our molecular data show that it belongs to *A. felis*. On our phylogenetic analyses, the partial *BenA* and/or *CalM* sequences of four other human isolates from clinical specimens identified previously as *A. viridinutans* signified identity with *A. felis* [33–34]. One of these (GM 02/39) was from a patient with IA and co-infection with *A. novofumigatus*, another *A. fumigatus* sibling species [8]. These findings are strongly supportive of a pathogenic role for *A. felis* in IA in humans. It is likely that analysis of other clinical isolates identified worldwide as *A. viridinutans* would reveal more strains of *A. felis*; determining the geographical distribution and prevalence of this species as an emerging fungal pathogen would be of interest. Our finding that *A. felis* was present in an air sample in Germany indicates that this species can be distributed by airborne propagules. This strain readily produced cleistothecia within 7 days after mating with CBS 130246 (*MAT1-1*).

Similar to chronic atypical IPA of humans, *A. felis* infection in feline invasive FRS has a protracted clinical course and spreads across anatomical planes to involve contiguous tissues (Figure 6). Domestic cats, the most common host described with *A. felis* infection, represent a suitable model for translational research of naturally occurring IA caused by *A. fumigatus*-like molds in immunocompetent hosts. In contrast to disease in humans, mycoses such as aspergillosis and cryptococcosis typically cause rhinosinusitis rather than pulmonary infection in cats. This may be associated anatomical differences in the nasal cavity and paranasal sinuses resulting in preferential deposition of inhaled fungal spores within the sinonasal cavity in cats compared to the lower respiratory tract in humans. Most isolates in the current study were obtained from cats with sino-orbital aspergillosis that failed to respond to aggressive multimodality therapy including posaconazole, amphotericin-B and, in some cases, radical orbital debridement surgery (exenteration) [13]. The majority were euthanased due to disease progression with severe signs including blindness and generalised seizures. IPA in the two human patients from which *A. felis* was isolated, was similarly refractory to treatment with posaconazole, voriconazole and caspofungin or liposomal amphotericin-B and caspofungin respectively [8,15]. Infections were fatal in both cases. In addition to *A. felis*, we have demonstrated that other species in the *Aspergillus* section *Fumigati* cause FRS in domestic cats including *A. fumigatus*, *A. udagawae*, *A. lentulus* and *N. pseudofischeri*.

Epidemiological cut off values (ECV) of amphotericin B for several *Aspergillus* species have recently been determined using the CLSI broth microdilution method [36]. The modal MIC of amphotericin B for 3, 988 *A. fumigatus* isolates was 0.5 μg/mL and the ECV that captured 95% of the modelled wild-type population was ≤2 μg/mL. MICs of amphotericin B were ≤1 μg/mL for all 13 *A. felis* isolates tested in our study and were ≤0.5 μg/mL for 12 of these. By contrast, three other cryptic species *A. lentulus*, *A. udagawae* and *A. fumigatiaffinis* have comparatively high MICs of amphotericin B [4,34]. Elevated MICs of amphotericin B have been associated with poor clinical outcomes for infections caused by *A. terreus* and *A. udagawae* [35,37]. Including antifungal susceptibility data published previously for human *A. felis* isolates CM 5623, CM3147, CM4158 and GM 02/39, high MICs of voriconazole (4 μg/mL) and/or itraconazole (≥1 μg/mL) occurred in 41% (7/17) of *A. felis* isolates [8,15,34]. Similar susceptibility trends have been observed in *N. pseudofischeri* [34]. However, where azole cross resistance was identified in *A. fumigatus* isolates it was usually between itraconazole and posaconazole (54%) and was uncommon between itraconazole and voriconazole (7%) [38]. High MICs of itraconazole (>16 μg/mL) and voriconazole (4 μg/mL) correlated with clinical outcome in one case of *A. felis* IA in a human (isolate CM5623) where sequential administration of these failed to control disease progression [15]. Additionally, one feline isolate with a caspofungin MEC of 2 μg/mL in this study, along with two human isolates with MEC of 1 μg/mL reported previously [8,34] suggests variable susceptibility of *A. felis* to caspofungin *in vitro*. Caspofungin ECVs accounting for 95% and 99% of the modelled *A. fumigatus* population were set at 0.5 μg/mL and 1 μg/mL, respectively [39]. The antifungal susceptibility profile of *A. felis* indicates that it is another *A. fumigatus*-like mold with *in vitro* resistance to antifungal agents used routinely for prophylactic therapy and treatment of IA.

Conclusion

A. felis is an important new species in *Aspergillus* section *Fumigati* applying a polyphasic taxonomical approach including molecular, morphological, physiological and ecological data. *A. felis* is an emerging agent of invasive aspergillosis in cats, dogs and humans.

Supporting Information

Figure S1 Phylogenetic analysis of the ITS gene for *A. felis* sp. nov isolates and other closely related species as conducted in MEGA5 [22] showing best scoring maximum parsimony (MP) trees constructed using the close-neighbor-interchange algorithm [40]. Bootstrap percentages of the MP analysis are presented at the nodes for values >70%. Trees are drawn to scale, with branch lengths calculated using the average pathway method, expressed in units of the number of changes over the whole sequence.
(TIF)

Table S1 Genbank accession numbers for genes of additional isolates used in morphologic and/or phylogenetic analyses in this study.
(DOC)

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Author Contributions

Conceived and designed the experiments: VRB TMVD JH JV RAS. Performed the experiments: VRB TMVD SEK PM. Analyzed the data: VRB TMVD JH SEK PM JV RAS. Contributed reagents/materials/analysis tools: VRB TMVD JH SEK PM MDP MR JV RAS. Wrote the paper: VRB TMVD JH SEK PM MDP MR JV RAS.

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4.3 Further discussion and conclusions

In describing this new species we apply the revised taxonomic approach, that is, the one-fungus one-name principal outlined in Chapter 2. In doing so, we are amongst the first mycologists to apply this controversial revision (Houbraken et al., 2014). The growth temperature studies that I performed show that *A. felis* can be distinguished from the related species *A. viridinutans* by its ability to grow at 45 °C and from *A. fumigatus* by its inability to grow at 50 °C. This information has application for mycologists with regard to discerning cryptic and non-cryptic species isolated from clinical samples and sparked the following comment from Prof. Maiken Cavling Arendrup, MD, PhD, Chairman of The EUCAST Antifungal Susceptibility Testing Subcommittee and President of the Nordic Society for Medical Mycology, after our manuscript was posted on the ProMED-mail list-serve of the international society of infectious diseases:

"Whilst reliable species identification of these cryptic species requires molecular techniques, separation of *A. fumigatus* from the other species is possible simply by taking advantage of the unique thermo-tolerance of *A. fumigatus* ; that is, *A. fumigatus* but not the others, is able to grow at high temperatures in the range of 48-50 deg C or higher. At SSI [Statens Serum Institut] this is systematically done for all *A. fumigatus* complex isolates and if not confirmed as *A. fumigatus* sensu stricto by this procedure, molecular identification is adopted. Following this procedure no *A. felis* has been detected so far suggesting it is currently a rare pathogen. If this procedure may misidentify some isolates when 48 deg C rather than 50 deg C is used (and which is the current standard at many reference laboratories in Europe) is however not evident from the current PLoS ONE publication."

Further information has since come to light in a recent publication in which another novel species was discovered in *Aspergillus* Section Fumigati, specifically in the *Aspergillus viridinutans* complex (Novakova et al., 2013). The *A. viridinutans* complex now includes five species, *A. viridinutans, A. udagawae, A. wyomingensis*, *A. felis* and*, A. aureolus.* Novakova and others found that all *A. viridinutans* complex isolates tested

had maximum growth temperatures between 42°C and 45°C and no isolates grew at 47°C. Therefore, for those laboratories using failure of growth at 48°C to discern cryptic from non-cryptic isolates, this technique should reliably distinguish *A. viridinutans* complex members from *A. fumigatus* sensu stricto. Like all morphological methods for fungal identification, results of growth temperature studies should be interpreted with caution. The authors of a guest editorial on our publication in Chapter three identified an *A. fumigatus* strain, confirmed by molecular methods, that was unable to grow at 50°C (Garcia et al., 2012a).

With the application of molecular tools for fungal identification, cryptic species are being increasingly detected as pathogens, e.g. *A. udagawae* as outlined in Chapter 2. (Gyotoku et al., 2012; Sugui et al., 2010; Vinh et al., 2009b). The frequency of A*. felis* as a pathogen amongst human hosts is yet to be elucidated. Cryptic species are thought to account for approximately 5% of cases of IA in humans. In this research study we identified *A. felis* from morphological and molecular analyses as a cause of IA in one human host. We also determined that several sequences of human isolates from patients with aspergillosis that had been deposited on GenBank as *A. viridinutans* were actually *A. felis*. More recently new isolates have been identified on similar analyses of sequences deposited in GenBank (Novakova et al., 2013). Retrospective molecular studies of archived fungal cultures and clinical specimens from patients with aspergillosis to determine the frequency of cryptic species as pathogens including *A. viridinutans* complex members as pathogens, is warranted in Australia.

Chapter 5. Computed Tomographic Features of Feline Sino-nasal and Sino-orbital Aspergillosis.

5.1 Background

In Chapter two I described the clinicopathological features of FURTA. At the end of case recruitment for that study in 2009 computed tomographic (CT) findings were available for only a small cohort of cases due to the limited availability of on-site veterinary CT. In 2008 the Valentine Charlton Cat Centre (VCCC) installed an on-site CT which increased the ability to recruit cases for study. Between 2007 and 2012 16 cases were recruited prospectively through a multi-centre study, including eight cases with on-site CTs performed at the VCCC. At the time this study was submitted for publication, descriptions of CT features of FURTA were limited to several case reports and one case series of cats with URTA that did not recognise the two anatomical forms of disease (SNA and SOA) (Karnik et al., 2009). The objectives of this study were to describe the CT features of URTA, to determine whether cats with SOA have concurrent involvement of the sinonasal cavity, and to determine if there was any association between CT features and the anatomic form of aspergillosis and/or infecting species.

5.2 Main article

Barrs VR, Beatty JA, Dhand NK, Talbot JJ, Bell E, Abraham LA, Chapman P, Bennett S, van Doorn T, Makara M. Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. *The Veterinary Journal* 2014 Feb 28. doi: 10.1016/j.tvjl.2014.02.020. [Epub ahead of print].

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Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis

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ABSTRACT

Feline upper respiratory tract aspergillosis (URTA) occurs as two distinct anatomical forms, namely, sino-nasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA). An emerging pathogen, *Aspergillus felis*, is frequently involved. The pathogenesis of URTA, in particular the relationship between the infecting isolate and outcome, is poorly understood. In this study, computed tomography was used to investigate the route of fungal infection and extension in 16 cases (SNA $n = 7$, SOA $n = 9$) where the infecting isolate had been identified by molecular testing.

All cases had nasal cavity involvement except for one cat with SNA that had unilateral frontal sinus changes. There was a strong association between the infecting species and anatomic form ($P = 0.005$). *A. fumigatus* infections remained within the sino-nasal cavity, while cryptic species infections were associated with orbital and paranasal soft-tissue involvement and with orbital lysis. Cryptic species were further associated with a mass in the nasal cavity, paranasal sinuses or nasopharynx. Orbital masses showed heterogeneous contrast enhancement, with central coalescing hypoattenuating foci and peripheral rim enhancement. Severe, cavitated turbinate lysis, typical of canine SNA, was present only in cats with SNA. These findings support the hypothesis that the nasal cavity is the portal of entry for fungal spores in feline URTA and that the route of extension to involve the orbit is via direct naso-orbital communication from bone lysis. Additionally, a pathogenic role for *A. wyomingensis* and a sinolith in a cat with *A. udagawae* infection are reported for the first time.

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Introduction

Feline upper respiratory tract aspergillosis (URTA) was first described in 1982 (Wilkinson et al., 1982) and is now being increasingly reported (Kano et al., 2008, 2013; Barachetti et al., 2009; Furrow and Groman, 2009; Karnik et al., 2009; Giordano et al., 2010; Smith and Hoffman, 2010; Barrs et al., 2012; Declercq et al., 2012). Disease occurs over a wide geographic range, including Australia, the USA, the UK, mainland Europe, and Japan (Goodall et al., 1984; Tomsa et al., 2003; Smith and Hoffman, 2010; Barrs et al., 2012, 2013; Declercq et al., 2012; Kano et al., 2013). Two anatomic forms have been reported, namely, sino-nasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA; Barrs et al., 2012). The environmental saprophytic fungi that cause these infections are most commonly from the *Aspergillus fumigatus* complex, including *A. fumigatus* and *A. felis*, a closely related, recently discovered cryptic (*A. fumigatus*-mimetic) species (Kano et al., 2008, 2013; Barrs et al., 2012, 2013; Whitney et al., 2013).

SNA and SOA have dramatically different prognoses (Barrs and Talbot, 2014), but the determinants of these alternate outcomes are poorly understood. It is suspected that the nasal cavity (NC) is the route of infection in all cases and that SOA is an extension of NC infection, but systematic imaging studies to support this have not been conducted (Barrs et al., 2012). In humans with aspergillosis, invasion is generally associated with impaired host immunity rather than the virulence of the infecting fungal species (Romani, 2011). Data from feline cases suggest an influence of fungal species on pathogenesis; *A. fumigatus* infections are frequently associated with non-invasive sino-nasal cavity (SNC) infections, while a cryptic species, A. *felis*, causes invasive sino-orbital disease (Barrs et al., 2012, 2013).

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Table 1
Factors used for acquisition of computed tomographic images.

Centre[a]	Number of cases	Reconstructed slice thickness bone tissue algorithm (mm)	Reconstructed slice thickness soft tissue algorithm (mm)	Beam collimation (number of data channels)	Detector row width (mm)	Tube potential (kV)	Tube current (mA)	Pitch factor
VCCC	8	1	3	16	0.75	120	200	0.4
RPAH	2	0.75	3	16	0.75	120	200	0.4
UMVCH	3	2	2	1	0.75	120	100	1
MUVH	2	2	2	2	0.75	130	120	1
VSEC	1	1	1	1	1	130	60	1

[a] VCCC, Valentine Charlton Cat Centre, Sydney, Australia; RPAH, Royal Prince Alfred Hospital, Sydney, Australia; UMVCH, University of Melbourne Veterinary Clinic and Hospital, Melbourne, Australia; MUVH, Murdoch Veterinary Hospital, Perth, Australia; VSEC, Veterinary Specialist and Emergency Centre, Levittown, Philadelphia, USA.

Descriptions of computed tomography (CT) findings in cats with URTA are limited to case reports (Halenda and Reed, 1997; Hamilton et al., 2000; Whitney et al., 2005; McLellan et al., 2006; Kano et al., 2008; Barachetti et al., 2009; Furrow and Groman, 2009; Giordano et al., 2010; Smith and Hoffman, 2010), and one case series of mycotic rhinitis that included five cats with URTA, but did not distinguish between SNA and SOA or identify the aetiologic agents (Karnik et al., 2009).

The objectives of the present study were to describe the CT features of URTA, to determine whether cats with SOA have concurrent involvement of the SNC, and to determine if there was any association between CT features and the form of aspergillosis and/or infecting species.

Materials and methods

CT examinations of the heads of cats with confirmed URTA from February 2007 to October 2012 were identified retrospectively. Criteria for the diagnosis of URTA were identification of fungal hyphae on cytology or histology of tissue biopsies, or sino-nasal fungal plaques and molecular identification of the isolate using 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). Signalment data were collected for review.

CT examinations were included if thin bone and soft-tissue transverse images of the skull obtained perpendicular to the soft palate were available for review. The examinations were performed at one of five centres (Table 1; Phillips Brilliance, 16 Slice, Phillips Medical Systems; Toshiba Aquilion, 16-slice, Toshiba Medical Systems Corporation; Toshiba X vision, 1-slice helical, Toshiba Medical Systems; Siemens Somatom Duo, 2-slice helical scanner; Siemens Esprit plus, 1-slice helical scanner, Siemens, Medical Solutions). The field of view was adjusted to the individual skull dimensions and a 512 × 512 matrix was used.

CT scans were reviewed by a Board certified radiologist (MM), blinded to isolate identity and clinical findings. Where more than one examination had been performed, only the first was reviewed. Non-ionic iodinated contrast was administered at 660 mg/kg iodine IV (Omnipaque, GE Healthcare). Standard bone and soft-tissue window algorithms were used (Table 1). Images were reviewed using standardised bone (window width, 4500 HU; window level, 1100 HU) and soft-tissue (window width, 400 HU; window level, 40 HU) display settings.

Cases were assigned to one of two groups, SOA or SNA, based on the presence or absence of a retrobulbar mass, respectively. Criteria for CT evaluation, adapted from a previous study (Karnik et al., 2009), included the amount and laterality of soft-tissue attenuation within the SNC, extension of lesions within the nasal cavity, lysis and/or sclerosis of the bones forming the NC (turbinates, septum, nasal bone and adjacent maxilla) and paranasal bones (frontal bone, orbital bone [orbital lamina], palatine bone, zygomatic bone, tympanic bullae and cribriform plate), nasopharyngeal (NP) involvement, paranasal soft-tissue involvement, and lymph-node involvement.

The severity of NC lesions was graded as none, minimal (affecting less than one-third), moderate (affecting two-thirds), or severe (affecting more than two-thirds). The NC was divided into rostral (rostral to teeth 104 and 204), middle (extending from teeth 104 and 204 to 108 and 208), and caudal (caudal to teeth 108 and 208) regions. Lysis, soft-tissue attenuation and sclerosis of the paranasal bones were graded as none, minimal, moderate, or severe. The pattern of contrast enhancement was described as none, normal mucosal enhancement, homogeneous, heterogeneous, or peripheral.

Statistical analyses

Frequency tables were prepared for all variables. Associations between the two anatomic forms of URTA and the respective aetiologic agent identified (*A. fumigatus* or cryptic species), and between anatomic form and variables indicative of soft-tissue attenuation, lysis and reactive bone lesions in nasal and paranasal regions, were evaluated using contingency tables and Fisher's exact tests. Variables representing severity were collapsed into two categories, (1) severe and (2) non-severe, for statistical analyses, because of the limited number of observations in each class. A 5% level of significance was used and all *P* values were two sided. Analyses were conducted using commercially available software (SAS 2002–2003).

Results

Sixteen cats met the inclusion criteria, including seven cats with SNA and nine with SOA. Post-contrast images were available in 13 cases (six SNA, seven SOA). One cat with SOA that had orbital exenteration before CT was excluded from statistical analyses of orbital CT features. Signalment data and molecular identity of fungal pathogens are presented in Table 2.

Isolates not reported previously were *A. fumigatus* (two SNA; Cases 2 and 5), *A. felis* (two SOA; Cases 11 and 13), *A. udagawae* (two SOA; Cases 14 and 15) and *A. wyomingensis* (one SOA; Case 16). One cat with *A. fumigatus* infection was co-infected with *Scedosporium aurantiacum* (Case 5). Anatomic form was highly associated with infecting species; of cats with SNA, five were infected with *A. fumigatus* and two with cryptic species, while all nine cats with SOA were infected with cryptic species ($P = 0.005$; Table 3). CT findings significantly associated with cryptic species infections included the presence of a mass in the NC, frontal sinus (FS), sphenoid sinus (SS) or NP; paranasal soft-tissue attenuation; orbital soft-tissue attenuation; and orbital bone lysis (Table 3).

CT findings, including frequency and severity of soft-tissue attenuation and bone lysis in the NC, nasopharynx, paranasal sinuses and tympanic bullae, are presented in Table 4. All cats had NC involvement, including increased soft-tissue attenuation and turbinate lysis, except one cat with SNA that had unilateral FS changes only. Of three cases with cryptic infections and focal soft-tissue NC masses, there was homogeneous enhancement in the two cases in which contrast medium was administered. Severe turbinate lysis occurred only in cats with SNA (three *A. fumigatus*, one *A. felis*; $P = 0.005$; Fig. 1). FS involvement was unilateral in 12/13 cases and was ipsilateral to the affected orbit in five cats with SOA. FS changes were characterised by soft tissue opacification without enhancement, consistent with fluid or poorly vascularised tissue in 11 cases.

FS attenuation was more severe in cats with SOA ($P = 0.040$; Table 4). One case of SNA and *A. fumigatus* infection had moderate thickening of the FS mucosa with homogenous contrast enhancement, while another with SOA had a soft-tissue attenuating mass within the FS (Fig. 2). No contrast study was available for the latter. SS soft-tissue attenuation was accompanied by ipsilateral ($n = 8$) or bilateral ($n = 1$) FS involvement. SS changes were consistent with fluid in seven cases and with a mass in two cats with SOA. One mass showed central heterogeneous contrast enhancement and

Table 2
Signalment, aetiologic agent and anatomic form of aspergillosis.

Case	Age (years)	Sex[a]	Breed[a]	Form[a]	Species	GenBank accession numbers		
						ITS[a]	*benA*[a]	*CalM*[a]
1[b]	6.8	F	Ragdoll	SNA	*A. fumigatus*	–	–	–
2	8	F	SSH	SNA	*A. fumigatus*	–	–	–
3[b]	11	F	Persian	SNA	*A. fumigatus*	–	–	JX021722
4[b]	7.4	M	Persian	SNA	*A. fumigatus*	–	–	–
5	2.8	F	DSH	SNA	*A. fumigatus/Scedosporium aurantiacum*	–	–	–
6[b]	14.8	F	DLH	SNA	*A. lentulus*	–	–	JX021720
7[b]	13	M	DLH	SNA	*A. felis*	JX021681	JX021704	JX021724
8[b]	5	F	CR	SOA	*A. felis*	JX021675	JX021694	JX021717
9[b]	2	M	HIM	SOA	*A. felis*	JX021682	JX021705	JX021725
10[b]	2	F	DSH	SOA	*A. felis*	JX021683	JX021706	JX021726
11	2.3	F	DSH	SOA	*A. felis*	–	–	–
12[b]	3.6	M	DSH	SOA	*A. felis*	KF558318	JX021700	JX021715
13	2.8	M	BSH	SOA	*A. felis*	KF703495	KF703496	–
14	4.1	M	Persian	SOA	*A. udagawae*	–	KF703497	KF703498
15	4.7	M	DSH	SOA	*A. udagawae*	–	–	KF703499
16	1.5	M	BSH	SOA	*A. wyomingensis*	JX021685	JX021709	–

[a] F, female (neutered); M, male (neutered); SSH, Scottish Shorthair; DSH, Domestic Shorthair; DLH, Domestic Longhair; CR, Cornish Rex; HIM, Himalayan; BSH, British Shorthair; SNA, sino-nasal aspergillosis; SOA, sino-orbital aspergillosis; ITS, internal transcribed spacer; *benA*, betatubulin; *CalM*, calmodulin.
[b] Signalment and molecular identification reported previously (Barrs et al., 2012, 2013).

Table 3
Associations between aetiologic agent and anatomic form or computed tomographic features in cats with URTA.

	Categories	*A. fumigatus* frequency (%)	Cryptic species frequency (%)	*P*
Anatomic form	SNA[a]	5 (71)	2 (29)	0.005
	SOA[a]	0 (0)	9 (100)	
NC, FS, SS or NP mass	Yes	0 (0)	8 (100)	0.026
	No	5 (63)	3 (37)	
Paranasal soft-tissue involvement	Yes	0 (0)	10 (100)	0.001
	No	5 (83)	1 (17)	
Orbital soft-tissue involvement	Yes	0 (0)	9 (100)	0.002
	No	5 (83)	1 (17)	
Orbital bone lysis	Yes	2 (17)	10 (83)	0.022
	No	3 (100)	0 (0)	

NC, nasal cavity; FS, frontal sinus; SS, sphenoid sinus; NP, nasopharyngeal.
[a] SNA, sino-nasal aspergillosis; SOA, sino-orbital aspergillosis.

homogeneous peripheral enhancement. The other mass was a calcified density of 1835 HU, consistent with a sinolith, identified in a cat with A. *udagawae* infection (Fig. 3).

Of the 10 cats with increased soft-tissue attenuation of the nasopharynx (Table 4), five had homogeneous contrast enhancement of NP mucosa only, most consistent with fluid. Five other cats with cryptic infections (four SOA; one SNA, *A. lentulus*) had an NP mass, with heterogeneous and homogeneous enhancement, respectively, in the two cats with SOA in which contrast was administered.

Tympanic bullae changes were seen in two cases of SNA without NP involvement (Table 4). One showed minimal bullous effusion on the left side in the case with right FS involvement only. The other had bilateral NC involvement and a minimal

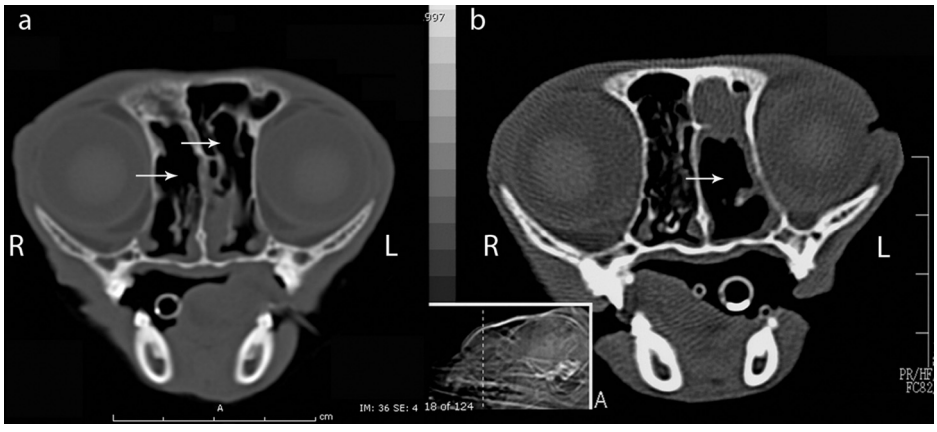

Fig. 1. Transverse skull CT images (bone algorithm) of two cats with SNA due to *A. fumigatus* infection showing severe 'cavitated' turbinate lysis (a) bilaterally (arrows) and (b) in the left nasal cavity.

Table 4
Soft-tissue attenuation and bone lysis in the nasal cavity, nasopharynx, paranasal sinuses and tympanic bullae on computed tomography in 16 cats with upper respiratory tract aspergillosis.

	Categories	SNA frequency (%)	SOA frequency (%)	Total	*P*[a]
Type of sino-nasal disease	Bilateral	5 (38)	8 (62)	13	0.55
	Unilateral	2 (67)	1 (33)	3	
Type of bilateral involvement	Asymmetric	4 (36)	7 (64)	11	1.0
	Symmetrical	1 (50)	1 (50)	2	
Nasal region involved	Rostral to caudal	4 (44)	5 (56)	9	1
	Middle	1 (33)	2 (67)	3	
	Middle to caudal	1 (33)	2 (67)	3	
	Caudal	1 (100)	0 (0)	1	
Nasal cavity soft-tissue attenuation	None	1 (100)	0 (0)	1	0.162
	Minimal	1 (33)	2 (67)	3	
	Moderate	3 (60)	2 (40)	5	
	Severe	2 (29)	5 (71)	7	
Turbinate lysis	None	1 (100)	0 (0)	1	0.005
	Minimal	2 (33)	4 (67)	6	
	Moderate	0 (0)	5 (100)	5	
	Severe	4 (100)	0 (0)	4	
Nasal septum lysis	Minimal	1 (50)	1 (50)	2	0.55
	Moderate	1 (100)	0 (0)	1	
Cribriform plate lysis	Minimal	1 (33)	2 (67)	3	0.213
	Moderate	1 (100)	0 (0)	1	
Nasal bone or adjacent maxilla lysis	None	3 (33)	6 (67)	9	0.615
	Minimal	2 (40)	3 (60)	5	
	Moderate	2 (100)	0 (0)	2	
Frontal sinus soft-tissue attenuation	None	1 (33)	2 (67)	3	0.040
	Minimal	1 (100)	0 (0)	1	
	Moderate	4 (80)	1 (20)	5	
	Severe	1 (14)	6 (86)	7	
Sphenoid sinus soft-tissue attenuation	None	4 (57)	3 (43)	7	0.789
	Minimal	0 (0)	1 (100)	1	
	Moderate	1 (100)	0 (0)	1	
	Severe	2 (29)	5 (71)	7	
Nasopharyngeal soft-tissue attenuation	None	5 (83)	1 (17)	6	0.153
	Minimal	0 (0)	3 (100)	3	
	Severe	2 (29)	5 (71)	7	
Nasopharyngeal mass	No	6 (55)	5 (45)	11	0.585
	Yes	1 (20)	4 (80)	5	
Tympanic bulla involvement	No	5 (36)	9 (64)	14	0.175
	Yes	2 (100)	0 (0)	2	

[a] Variables representing severity were collapsed into two categories (severe and non-severe) for statistical analyses because of limited number of observations in each class.

soft-tissue attenuating mass in the bulla with homogenous contrast enhancement.

Reactive bony changes of the nasal and paranasal bones are presented in Table 5. Nasal bone sclerosis was always accompanied by sclerosis of the frontal or zygomatic bone.

Paranasal bone lysis and soft-tissue abnormalities are described in Table 6. Cryptic species infections ($n = 11$) were associated with orbital ($P = 0.002$) or paranasal soft-tissue involvement ($P = 0.001$; Table 3). Orbital masses were characterised by heterogeneous contrast enhancement, with central coalescing hypoattenuating foci and peripheral rim enhancement (Fig. 4). Masses were located in the rostral and ventromedial aspect of the orbit, causing dorsolateral displacement of the globe. The largest mass displaced the zygomatic arch laterally. Orbital masses typically extended rostrally to the rostral aspect of the zygomatic arch and caudally to the rostral aspect of the ramus of the mandible, immediately rostral to the temporomandibular joint. In all cases of SOA, lateral extension into the paranasal maxillary soft tissues was identified, resulting in

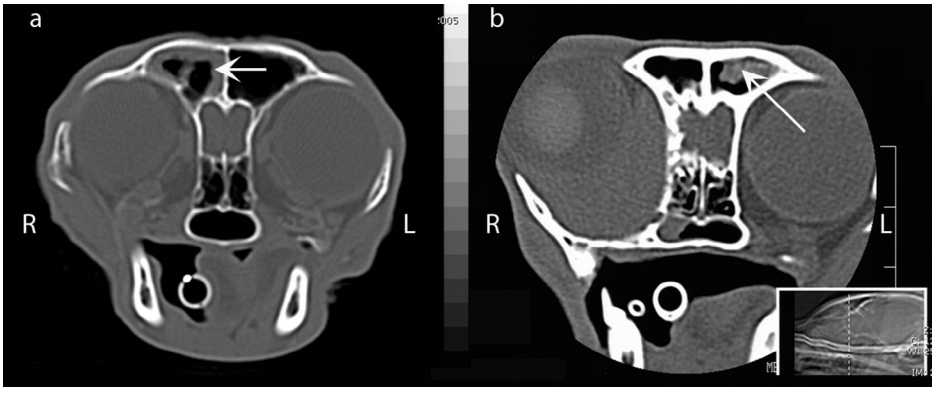

Fig. 2. Transverse skull CT images (bone algorithm) showing (a) right frontal sinus mucosal thickening in a cat with SNA and *A. fumigatus* infection (arrow) and (b) a soft-tissue attenuating mass in the left frontal sinus of a cat with SOA (right orbital mass) due to *A. felis* infection (arrow).

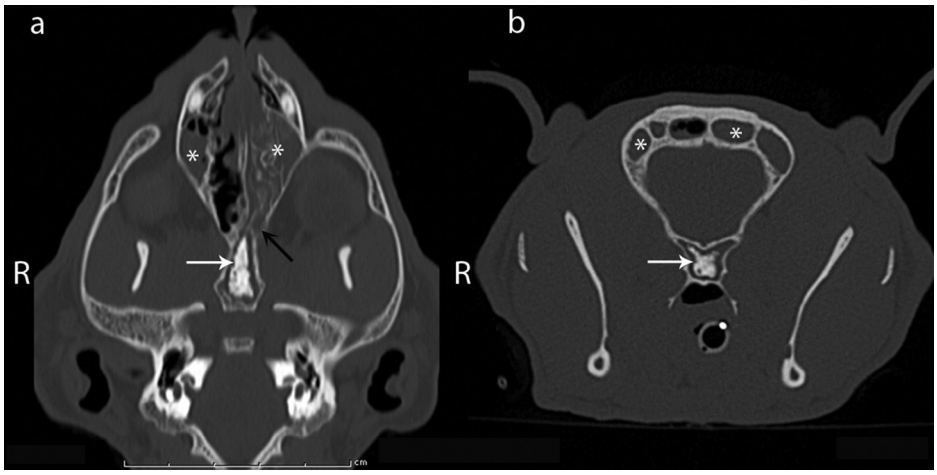

Fig. 3. (a) Dorsal and (b) transverse skull CT images (bone algorithm) showing a calcified density (1835 HU) in the sphenoid sinus of a cat with SOA and *A. udagawae* infection (white arrows). Note the soft-tissue attenuating material bilaterally (asterisks) in (a) the nasal cavity and (b) frontal sinuses; and (a) left orbital lysis (black arrow).

Table 5
Reactive bone lesions of the nasal and paranasal bones on computed tomography in 16 cats with upper respiratory tract aspergillosis.

	Categories	SNA frequency (row %)	SOA frequency (row %)	Total	P[a]
Nasal bone sclerosis	None	2 (22)	7 (77)	9	0.126
	Minimal	3 (60)	2 (40)	5	
	Moderate	2 (100)	0 (0)	2	
Frontal bone sclerosis	None	2 (29)	5 (71)	7	0.541
	Minimal	0 (0)	1 (100)	1	
	Moderate	3 (60)	2 (40)	5	
	Severe	2 (67)	1 (33)	3	
Zygomatic bone sclerosis	None	7 (50)	7 (50)	14	0.475
	Moderate	0 (0)	2 (100)	2	
Cribriform plate sclerosis	None	6 (40)	9 (60)	15	0.438
	Moderate	1 (100)	0 (0)	1	

[a] Variables representing severity were collapsed into two categories (severe and non-severe) for statistical analyses because of limited number of observations in each class.

a distinct mass effect in eight cases. Orbital masses extended ventrally into pterygopalatine fossa of the oral cavity in six cases (Fig. 4). Soft-tissue attenuation adjacent to an area of complete lysis of the nasal bone was present in one cat with SNA due to *A. felis* infection.

Orbital bone lysis was detected in both anatomic forms and was significantly associated with aetiologic agent (*A. fumigatus* v cryptic species) (Tables 3 and 4). Orbital lysis was complete in four cases (SNA and SOA, $n = 2$ each) and punctate in eight cases (SNA and SOA, $n = 2$ each; Fig. 5). Of the four cats with frontal bone lysis,

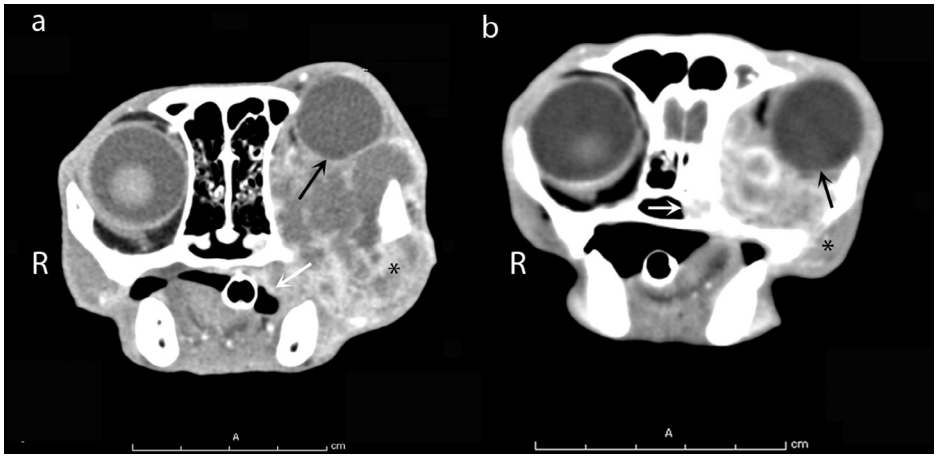

Fig. 4. Transverse post-contrast soft-tissue images of the head reconstructed using a soft-tissue algorithm showing left orbital masses in two cats with SOA and *A. felis* infection. There is heterogeneous contrast enhancement, with central coalescing hypoattenuating foci and peripheral rim enhancement. There is (a and b black arrow) compression and dorsal displacement of the globe, and (a, white arrow) extension into the oral cavity; (b, white arrow) nasopharynx, and (a and b, black asterisk) adjacent paranasal maxillary soft tissues.

Table 6
Paranasal bone lysis and paranasal soft-tissue abnormalities in 16 cats with upper respiratory tract aspergillosis.

	Categories	SNA frequency (%)	SOA frequency (%)	Total	*P*[a]
Unilateral orbital soft-tissue attenuation	None	7 (100)	0 (0)	7	0.0002
	Severe	0 (0)	8 (100)	8	
Paranasal soft-tissue attenuation (facial)	None	6 (100)	0 (0)	6	0.003
	Mild	0 (0)	1 (100)	1	
	Moderate	1 (100)	0 (0)	1	
	Severe	0 (0)	8 (100)	8	
Paranasal soft-tissue (facial) mass	No	7 (88)	1 (12)	8	0.001
	Yes	0 (0)	8 (100)	8	
Pterygopalatine fossa mass	No	7 (70)	3 (30)	10	0.011
	Yes	0 (0)	6 (100)	6	
Orbital bone lysis	No	3 (100)	0 (0)	3	0.077
	Yes	4 (33)	8 (67)	12	
Orbital bone lysis	None	3 (100)	0 (0)	3	0.231
	Minimal	0 (0)	1 (100)	1	
	Moderate	3 (43)	4 (57)	7	
	Severe	1 (25)	3 (75)	4	
Type of orbital bone lysis	Unilateral	0 (0)	6 (100)	6	0.061
	Bilateral	4 (67)	2 (33)	6	
Frontal bone lysis	None	4 (33)	8 (67)	12	1
	Minimal	2 (100)	0 (0)	2	
	Moderate	1 (50)	1 (50)	2	
Palatine bone lysis	None	4 (33)	8 (67)	12	0.262
	Minimal	2 (100)	0 (0)	2	
	Moderate	0 (0)	1 (100)	1	
	Severe	1 (100)	0 (0)	1	
Zygomatic bone lysis	None	7 (54)	6 (46)	13	0.213
	Minimal	0 (0)	2 (100)	2	
	Moderate	0 (0)	1 (100)	1	
Mandibular lymph node enlargement	Not scanned	0 (0)	1 (100)	1	0.011
	Minimal	4 (100)	0 (0)	4	
	Moderate	3 (50)	3 (50)	6	
	Severe	0 (0)	5 (100)	5	
Retropharyngeal lymph node enlargement	Not scanned	1 (33)	2 (67)	3	0.135
	None	3 (100)	0 (0)	3	
	Minimal	2 (50)	2 (50)	4	
	Moderate	0 (0)	3 (100)	3	
	Severe	1 (33)	2 (67)	3	

[a] Variables representing severity were collapsed into two categories (severe and non-severe) for statistical analyses because of limited number of observations in each class.

two with SNA also had palatine bone lysis (Table 5). Frontal, palatine and zygomatic bone lysis was punctate in all cases, except for one cat that had SOA and a focal area of severe complete palatine lysis. Severe mandibular lymph node enlargement was documented only in cases of SOA ($P = 0.011$). All enlarged lymph nodes enhanced homogeneously.

Discussion

This study of CT findings in feline sino-nasal and sino-orbital aspergillosis documented involvement of the SNC in all cases, regardless of the anatomic form. This supports the hypothesis that orbital involvement in feline SOA occurs subsequent to NC infec-

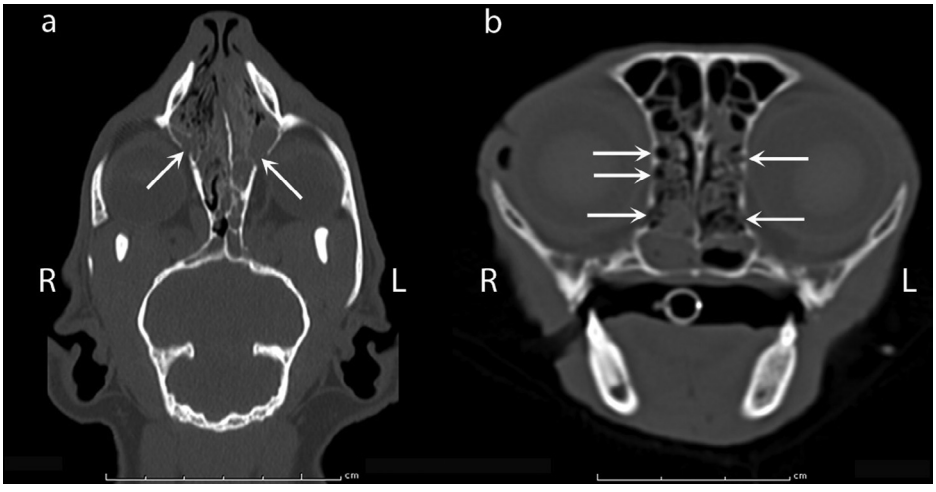

Fig. 5. (a) Reconstructed dorsal and (b) transverse skull CTs showing regions of severe complete bilateral orbital lysis (arrows) in a cat with SNA due to *A. felis* (a) and bilateral moderate punctate lysis (arrows) in a cat with SNA due to *A. entulus* (b).

tion following inhalation of *Aspergillus* conidia, but evaluation of serial CTs over time would be required to definitively demonstrate extension of disease from the NC to the orbit. Upper respiratory tract signs can be absent at the time of diagnosis when exophthalmos is present (Smith and Hoffman, 2010; Barrs et al., 2012), but the extent of SNC involvement should be evaluated, as this might inform therapeutic decisions. All cats with SOA had evidence of naso-orbital communication due to lysis of the orbital lamina, identifying direct extension as the most likely route of orbital involvement. This is supported by findings on CT, surgery or at post mortem in other cases, and by the fact that haematogenous spread has not been reported (Wilkinson et al., 1982; Hamilton et al., 2000; Barachetti et al., 2009; Giordano et al., 2010; Barrs et al., 2012). Orbital extension directly from the frontal sinus has been documented in one case (Halenda and Reed, 1997).

Interestingly, orbital bone lysis was common, regardless of anatomic form. In dogs with SNA due to *A. fumigatus*, orbital lysis is common, but SOA is rare (Saunders et al., 2002). Thus, additional factors are clearly necessary for the establishment of mycotic orbital disease in cats. Previous studies have found that *A. fumigatus* infections were confined to the SNC, while cases of SOA were caused by cryptic species, particularly *A. felis* (Kano et al., 2008, 2013; Declercq et al., 2012; Barrs et al., 2013; Barrs and Talbot, 2014). Imaging findings presented here are consistent with this putative role for fungal species in disease outcome. Species-specific fungal virulence factors could be involved in the establishment of invasive orbital disease. SOA in cats is similar to chronic granulomatous fungal rhinosinusitis in immunocompetent humans. This is an uncommon disease reported in Africa and Asia and caused by *A. flavus*, which has a propensity to colonise the nasal and paranasal sinuses in hot, dry climates (Thompson and Patterson, 2012). Similar to humans and dogs with SNA, nasal and paranasal bone lysis in feline URTA is probably due to a pro-inflammatory response by the host and fungal virulence factors, such as dermonecrolytic toxins (Peeters and Clercx, 2007; Thompson and Patterson, 2012). Fungal osteomyelitis has not been reported in feline URTA.

In addition to SNC involvement, CT features common to both anatomic forms included unilateral involvement of paranasal sinuses, and paranasal bone lysis and/or sclerosis. Cribriform plate lysis, a relative contraindication for topical antifungal treatment in canine SNA (Day, 2009), was detected in 25% of cases, highlighting the importance of CT evaluation in treatment planning. Some CT features were associated with infecting species and/or anatomic form. Orbital masses, abnormal paranasal soft-tissue attenuation and a mass effect in the NC, FS, SS or NP were only associated with cryptic species, while severe 'cavitated' lysis was only present in cats with SNA, including three cats with *A. fumigatus* infection. In dogs, *A. fumigatus* is the aetiologic agent of SNA in 98% of cases (Talbot et al., 2014). Characteristic CT features in canine cases include cavitated turbinate lysis, a rim of soft tissue of variable thickness along bones in the FS, maxillary recess and nasal passages, abnormal presence of soft-tissue accumulation and reactive bony change (Saunders et al., 2002, 2004; Saunders and Van Bree, 2003). Isolated FS involvement, documented in one cat in this study, has also been reported in canine SNA (Johnson et al., 2006).

Post-contrast features of orbital masses are similar to those reported in SOA in humans and in mycotic rhinosinusitis due to non-*Aspergillus* spp., including cryptococcosis, in cats, (Sivak-Callcott et al., 2004; Karnik et al., 2009; Pushker et al., 2011). Central hypoattenuating foci have been interpreted as areas of necrosis and peripheral enhancement as inflammatory response (DeLone et al., 1999; Almutairi et al., 2009). A similar pattern of enhancement can be seen with neoplasia in humans, where peripheral enhancement has been interpreted as well-perfused, viable tumour tissue (Nino-Murcia et al., 2000). Whether post-contrast features will aid in differentiating SOA from neoplasia in feline cases requires further characterisation of neoplastic changes in cats.

Previous retrospective analyses of CT findings in feline sino-nasal disease included few cases of mycotic rhinosinusitis (0–5%; Schoenborn et al., 2003; Tromblee et al., 2006). CT features associated with sino-nasal neoplasia included lysis of the paranasal bones, especially bilateral orbital bone lysis, moderate to severe turbinate destruction, lysis of the nasal septum, a homogeneous mass within the sino-nasal cavity, unilateral abnormal soft tissue or fluid within the frontal or sphenoid sinuses, and extension of the disease process into the orbit or paranasal facial soft tissues (Schoenborn et al., 2003; Tromblee et al., 2006). Interestingly, these were all features of aspergillosis in our study.

Changes in the tympanic bulla were rare and likely incidental in the present study. In contrast, one study reported that 28% of cats with sino-nasal neoplasia or inflammatory disease had bulla effusion secondary to auditory tube obstruction or ascending infection. However, only 1/46 cats in that study had sino-nasal disease of fungal aetiology (Detweiler et al., 2006).

Here we report sinolithiasis for the first time in feline URTA. Rhinolithiasis has been reported in one cat with SNA (Tomsa et al., 2003). Sinoliths, described in 50–75% of humans with maxillary sinus fungal mycetomas, are deposits of calcium-containing fungal metabolites in necrotic areas of mycelial plaques. Predisposing factors include chronic inflammation, impaired sinus drainage and the presence of a nidus of material of endogenous or exogenous origin, e.g. endodontic filling materials, around which calcification occurs (Lenglinger et al., 1996).

Molecular identification revealed two new isolates of *A. udagawae* from Australia and the USA. These, in addition to two previous isolates reported in Japan, establish *A. udagawae* as the second most common cause of feline SOA (Kano et al., 2008, 2013). *A. udagawae* is the most common cryptic species to cause invasive pulmonary aspergillosis in humans (Vinh et al., 2009). *A. wyomingensis*, reported here for the first time as a cause of feline SOA and as a pathogen, is a recently described soil saprophyte related to *A. felis* and to *A. udagawae* (Novakova et al., 2014). Accurate species identification is important, since cryptic species are more resistant to antifungal drugs than *A. fumigatus* (Barrs et al., 2013). Previous studies of feline URTA have demonstrated that breeds of cats with brachycephalic conformation are over-represented (Tomsa et al., 2003; Whitney et al., 2005; Barrs et al., 2012). These have included Himalayans, Persians, Exotic shorthairs, American shorthairs and Ragdoll cats. In this study, we additionally report disease in British and Scottish shorthair cats. Prospective studies are required to determine whether the increased risk of URTA in these breeds is genetic due to common ancestry, or if it is associated with SNC conformation combined with other factors such as viral upper respiratory tract infection (Barrs and Talbot, 2014).

The limitations of this study included the small sample size and its retrospective design, which precluded standardisation of CT conditions for each examination.

Conclusions

Common computed tomographic features of both anatomic forms of feline upper respiratory tract aspergillosis include asymmetric, bilateral sino-nasal cavity involvement, moderate to severe soft-tissue attenuation of the nasal cavity, sphenoid sinus and frontal sinus, moderate to severe turbinate lysis, and orbital bone lysis. A strong association was identified between the infecting fungal species and the anatomic form of the disease. Cryptic species were significantly associated with sino-orbital aspergillo-

sis, the presence of a mass in the nasal cavity, sinuses or nasopharynx, with paranasal soft-tissue attenuation, and orbital bone lysis.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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5.3 Conclusions

The findings from this study increase our understanding of the pathogenesis of URTA in cats. Extrapolating from other host species (e.g. humans) and considering the virulence factors of the infecting agent (i.e. air-borne infectious propagules) it is intuitive that orbital infections in cats are an extension of a sinonasal cavity infection subsequent to inhalation and colonization. Despite this, some authors have continued to discuss the possibility of retrobulbar infection via the conjunctiva with secondary "descending" nasal cavity infection (Giordano et al., 2010; Smith and Hoffman, 2010). Our systematic study shows that all cats with URTA have involvement of the sinuses and/or nasal cavity. To prove definitively that sinonasal colonisation occurs first in cats with SOA would require serial CTs over time in an experimental model of infection. However, as I established previously, in many cats with SOA clinical signs indicative of sinonasal cavity infection appear before up to six months before signs of orbital involvement. (Barrs et al., 2012a).

The CT findings that I have reported in this study are of clinical relevance to veterinary practitioners and also to diagnostic imaging specialists. The CT features of FURTA overlap those reported previously in cats with malignant neoplasia arising from the sinonasal cavity, including lymphoma and adenocarcinoma. This highlights the point that mycotic rhinosinusitis should be considered as a differential diagnosis in cats with CT findings consistent with neoplasia and where there is extension of the disease process from the nasal cavity to involve paranasal tissues including the orbit. An increased awareness of mycotic disease may make further investigations necessary to achieve definitive diagnosis more likely to be performed, including biopsy, serology and fungal cultures. Further information likely to be of use to those interpreting feline CTs is the finding that cats with SNA due to *A. fumigatus* infection, have similar CT features to those described in canine SNA including severe cavitated turbinate lysis.

Together with those cases described in Chapter 3, this work brings the number of cases of FURTA that I have published to 30. When analysed together with all other reported cases where molecular identification has been described, some interesting new epidemiological data emerge. With the two additional isolates of *A. udagawae*

described here we can now establish that the two most common causes of SOA in cats are *A. felis* and *A. udagawae,* both members of the *A. viridinutans* complex (Barrs et al., 2013b; Kano et al., 2008; Kano et al., 2013). Further, *A. udagawae* has a wide geographic distribution and has been documented to cause disease in human and feline hosts in Australia, the U.S.A and Japan (Gyotoku et al., 2012; Sugui et al., 2010; Vinh et al., 2009b). I also established a pathogenic role for first time, for the recently described *A. wyomingensis* (Novakova et al., 2013), the newest member of the *A. viridinutans* complex.

Chapter 6. Detection of *Aspergillus*-specific Antibodies by Agar Gel Double Immunodiffusion and IgG-ELISA in Feline Upper Respiratory Tract Aspergillosis.

6.1 Background

The sensitivity of serological tests to detect fungal antigen or *Aspergillus*-specific antibody in aspergillosis depends on the systemic immunocompetence of the host. Tests to detect fungal antigen, namely the cell wall antigen galactomannan, have a sensitivity of up to 90% in immunocompromised patients, including neutropenic human patients with pulmonary aspergillosis and dogs with disseminated invasive aspergillosis (DIA) (Garcia et al., 2012b; Maertens et al., 1999). However, these tests have a very low sensitivity (30% or less) in non-neutropenic humans with aspergillosis, in immunocompetent dogs with SNA, and in cats with URTA (Billen et al., 2009; Kitasato et al., 2009; Whitney et al., 2013b). Conversely, detection of *Aspergillus*-specific antibodies has a high test sensitivity of up to 90% in immunocompetent patients including dogs with SNA and humans with chronic pulmonary aspergillosis (Billen et al., 2009; Guitard et al., 2012; Ohba et al., 2012) while a detectable antibody response is mounted in less than 30% of immunocompromised patients including neutropenic humans with aspergillosis and dogs with DIA (Day et al., 1985; Hope et al., 2005b; Schultz et al., 2008).

The diagnostic value of serological tests to detect *Aspergillus*-antibodies in cats with URTA has not been evaluated. The objectives of this study were to determine if the sera of cats infected with cryptic species in *Aspergillus* section *Fumigati* cross react with antigens in a commercially available aspergillin derived from mycelia of *A. fumigatus, A. niger* and *A. flavus* and to assess the diagnostic value of detection of *Aspergillus*-specific antibodies using an agar-gel immunodiffusion (AGID) and IgG ELISA tests.

6.2 Main article

Barrs VR, Ujvari B, Dhand NK, Peters IR, Talbot J, Johnson LR , Billen F, Martin P, Beatty JA, Belov K., 2014. Detection of *Aspergillus*-specific antibodies by agar gel double immunodiffusion and IgG ELISA in feline upper respiratory tract aspergillosis. The Veterinary Journal: Manuscript accepted 21.12.14. doi:10.1016/j.tvjl.2014.12.020 [Epub before print].

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Detection of *Aspergillus*-specific antibodies by agar gel double immunodiffusion and IgG ELISA in feline upper respiratory tract aspergillosis

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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.

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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).

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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).

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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.

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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).

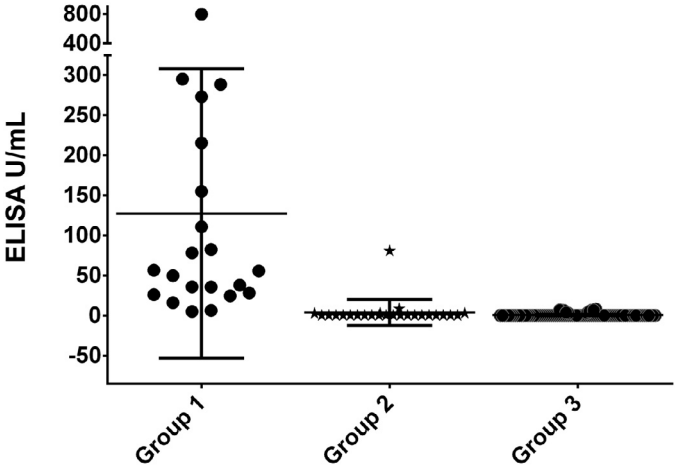

Fig. 1. ELISA units/mL for 21 group 1 sera (cats with aspergillosis; black circles), 25 group 2 sera (control cats with other upper respiratory tract disease; black stars) and 84 group 3 sera (control cats, either healthy or sick with non-respiratory disease; grey circles). Lines represent means ± standard 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.

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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.

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6.3 Conclusions

The results of this study prove that feline antibodies against the four most commonly isolated cryptic species in *Aspergillus* section *Fumigati* (*A. felis, A. udagawae, A. lentulus* and *A. thermomutatus/N. pseudofischeri*) cross react with a commercial aspergillin containing *A. fumigatus, A. niger* and *A. flavus.* This result was not unexpected, given the close phylogenetic relationship of cryptic species to *A. fumigatus* (Barrs et al., 2013a; Novakova et al., 2013). However, it was important to demonstrate this for the development of a diagnostic serological assay that relies on the use of a commercially available aspergillin that includes *A. fumigatus* mycelial antigens.

In chapter 3, a predilection for pure-bred cats with brachycephalic conformation was identified. In that study brachycephalic cats comprised 39% of the study population (Barrs et al., 2012a). Here for the first time, with the inclusion of two control groups comprising a group of cats with other upper respiratory tract disease, and a group of healthy cats and sick cats with non-fungal illnesses I was able to apply statistical analyses to demonstrate that cats with URTA were significantly more likely than all control groups to be pure-bred cats of brachycephalic conformation.

The qualitative AGID had poor sensitivity and high specificity, while the quantitative IgG ELISA had both high sensitivity and high specificity for the diagnosis of URTA. Thus, the Ig-G ELISA is a valuable non-invasive diagnostic tool for URTA in cats, and could readily be commercialized and made available to veterinary practitioners. Furthermore, the high prevalence of *Aspergillus*-specific antibodies in cats with URTA adds to the growing body of evidence that affected cats are systemically immunocompetent. We also systematically tested the sera of all cats with URTA in this study for the two of the most common viral causes of immunosuppression in cats, FIV (antibody) and FeLV (antigen) and found no cats with FeLV infection and a low prevalence of FIV-infection (4.76%) compared to a prevalence of 8 to 10% reported in domestic cat populations in Australia (Chang-Fung-Martel et al., 2013; Norris et al., 2007). There was also no evidence of other causes of systemic immunosuppression that have been documented

in cats with disseminated invasive aspergillosis, including feline panleukopenia virus infection, feline infectious peritonitis virus and prolonged corticosteroid therapy, in cats with URTA in this study.

Chapter 7. General Discussion

7.1 Background

The article included in this chapter was an invited review for *Veterinary Clinics of North America.* In it, I discuss my research findings in the wider context of other publications on upper respiratory tract aspergillosis around the time of submission of this thesis.

7.2 Summary of Results

This thesis presents the characterization of feline upper respiratory tract aspergillosis, including causative agents, aspects of pathogenesis, the spectrum of clinical disease, diagnosis, and treatment outcomes. The outcomes and results can be summarized as follows:

1. Cases of FURTA were recruited, forming the foundations of a case-bank and fungal isolate bank for future study (Chapters 3, 4, 5 and 6).

2. The clinical syndrome of FURTA was described. Two anatomic forms, SNA and SOA were recognised and fully characterised with regard to historical signs, clinical signs, and clinicopathological findings including haematological, serum biochemical, microbiological, histopathological, serological and computed tomographic findings. Haematological findings were non-specific. Hyperglobulinaemia was identified as the most common serum biochemical abnormality in cats with FURTA, and was only found in cats with SOA. Fungal culture of affected tissues in cats with URTA (sinonasal biopsies/fungal plaques/retrobulbar or pterygopalatine fossa biopsies) was found to be 95%. *Aspergillus* spp. clinical isolates from cats were found to grow on readily available commercial agar preparations including Sabouraud's dextrose agar and malt extract agar (Chapters 3, 5 and 6).

3. Computed tomographic features of FURTA were identified and described. CT features found to be common to both anatomic forms included bilateral, symmetrical SNC involvement and unilateral paranasal sinus involvement. Orbital masses, abnormal

paranasal soft-tissue attenuation and a mass effect in the NC, FS, SS or NP, were features of cryptic species infections and SOA. Severe cavitated lysis of the SNC was most commonly seen in cats *A. fumigatus* infections and SNA. Orbital masses were found to show heterogeneous enhancement after the administration of iodinated contrast and had central coalescing hypoattenuating foci with peripheral rim enhancement (Chapter 5).

4. The aetiological agents of FURTA were identified. *A. fumigatus* was found to be the most common cause of SNA in cats. Cryptic species within *Aspergillus* section *Fumigati,* and more specifically within the *A. viridinutans* complex, were found to be the most common cause of SOA in cats (Chapters 3, 4, 5, 6 and 7).

5. A novel species of *Aspergillus* within section *Fumigati* was discovered and named *A. felis.* The novel species was assigned on the basis of demonstration of morphological, biological and phylogenetic species concepts. *A. felis* was identified as the most frequent isolate from cats with SOA. Species within *Aspergillus* section *Nigri* (*A. niger*) and *Aspergillus* section *Flavi* (*A. flavus*) were identified as other aetiological agents of SNA in cats. A pathogenic role was ascribed to *A. wyomingensis* for the first time (Chapters 3, 4, 5, 6 and 7).

6. The novel species, *A. felis*, was identified as a pathogen in a dog with disseminated invasive aspergillosis and in a human with chronic invasive aspergillosis. Both infections were fatal. Antifungal susceptibility testing results of *A. felis* isolates from cats, dogs and humans identified resistance amongst some isolates to caspofungin, itraconazole, voriconazole or posaconazole. Amongst resistant isolates, cross-resistance to itraconazole and voriconazole, or to itraconazole, voriconazole and posaconazole was demonstrated. These results establish *A. felis* as another cryptic species with *in vitro* resistance to antifungal agents used routinely for prophylaxis and treatment of IA. (Chapter 4).

7. Multi-modality treatment of SOA was investigated using a tri-azole antifungal drug (itraconazole, posaconazole or voriconazole) alone, in combination with amphotericin-B

and terbinafine. Posaconazole was found to be well tolerated overall, with infrequent mild transient hepatic enzyme elevations. Voriconazole was associated with significant side-effects. The addition of radical debridement surgery (exenteration) to the treatment regime did not improve treatment outcomes (Chapter 3).

8. From post-mortem results, surgical exploration and computed tomographic findings, communications between the sinonasal cavity and orbit were identified in all cats with SOA. Furthermore, all cats with SOA were found to have sinonasal cavity involvement on computed tomographic examination. These findings are consistent with the nasal cavity as the portal of entry for *Aspergillus* spp. conidia in feline URTA and support that the route of extension to involve the orbit is via direct naso-orbital communication from bone lysis (Chapters 3 and 5).

9. Brachycephalic pure-bred cats of Persian lineage were identified to be predisposed to both anatomic forms of FURTA (Chapters 3, 5 and 6). Systemic immunosuppression was not identified in the vast majority of cats with FURTA. Common viral causes of immunosuppression including FIV, FeLV, FIP and feline panleukopenia virus infection were absent except in one case that was found to be infected with FIV. There was no history of previous corticosteroid usage or other immunosuppressive therapy prior to the onset of clinical signs in affected cats. The majority of cats with URTA had a robust IgG response to *Aspergillus* spp. infection (Chapter 3 and Chapter 6).

These results have improved our knowledge and understanding of the pathogenic role of cryptic species within *Aspergillus* section *Fumigati* in animals and humans.

7.3 Main article

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Feline Aspergillosis

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KEYWORDS

• Aspergillosis • Sinonasal aspergillosis • Sino-orbital aspergillosis • *Aspergillus felis*
• Fungal rhinosinusitis • Antifungals

KEY POINTS

- There are two forms of upper respiratory tract aspergillosis (URTA): sinonasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA). Both infections start in the nasal cavity, and SOA is the most common form (65% of cases).
- Brachycephalic breeds of cats, especially Persian and Himalayan, are predisposed to URTA.
- Feline SNA can be invasive or noninvasive. Noninvasive disease resembles SNA in dogs. The most common causes of SNA are *Aspergillus fumigatus* and *Aspergillus niger*.
- The most common cause of SOA is a recently described novel species, *A felis*, which is an *A fumigatus*-like fungus. Molecular identification is required to differentiate *A felis* from *A fumigatus*.
- The prognosis for SNA is favorable with topical antifungal therapy alone, or combined with systemic antifungals.
- Disseminated and non-URT focal forms of invasive aspergillosis are uncommon in cats, with little known about the etiologic agents. Young to middle-aged cats are affected. Concurrent immunosuppressive diseases have been identified in some cats.

INTRODUCTION

Aspergillosis is a mycosis of a diverse range of human and animal hosts including mammals and birds. Among the most common molds on earth, *Aspergillus* spp. are filamentous ascomycetes distributed primarily in soil and decaying vegetation that have an important role in recycling environmental carbon and nitrogen.[1] The genus is named after "aspergillum," a brush or implement with a perforated head used by Roman Catholic priests for sprinkling holy water, which resembles the fungi's spore-bearing conidial heads.[2]

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Feline upper respiratory tract aspergillosis (URTA) was first described in the early 1980s.[3] Of the more than 55 cases now reported, over two-thirds were described in the last 5 years.[3–16] Other forms of aspergillosis in cats including disseminated[5,17–21] and focal (non-URT) invasive infections[17,19,20,22–32] are reported less commonly and little is known about the etiologic agents.

CLASSIFICATION SCHEMES

Aspergillosis can be classified by body system involvement, duration of infection, pathology, and pathogenesis. Disease is defined as invasive if there is hyphal invasion into tissues.[33] The respiratory tract is the most common site of disease in humans and animals reflecting the primary inhalational route of infection.

Invasive aspergillosis (IA) in humans occurs predominantly in the sinopulmonary tract of immunocompromised individuals associated with inhalation of *Aspergillus* spp conidia, and invasive pulmonary aspergillosis accounts for more than 90% of IA cases.[34] URTA occurs less commonly and is classified as invasive or noninvasive fungal rhinosinusitis. The classification of sinopulmonary forms of aspergillosis in humans is summarized in **Table 1**.[35–38]

By contrast, URTA is the most common form of aspergillosis reported in mostly immunocompetent cats and dogs.[11,39] URTA can be further subdivided into sinonasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA). In dogs SNA accounts for more than 99% of cases and is noninvasive, whereas in cats SOA is the most common form and is invasive (65% of cases).[3–5,7,8,11,14,16,40–45]

Disseminated IA typically occurs in immunocompromised hosts and is defined as active infection in two or more noncontiguous sites or the hematogenous spread of disease.[35] There are few reports of disseminated IA in cats and most cases had pulmonary involvement.[17–21] Focal (non-URT) invasive infections have also been reported in cats involving lung,[17,19,20,22–25,28] gastrointestinal tract,[20,30–32,46] or urinary bladder.[26,27]

ETIOLOGY

Several hundred species have been ascribed to the genus *Aspergillus*, which includes four major subgenera; *Circumdati*, *Nidulantes*, *Fumigati*, and *Aspergillus*. Each subgenus comprises from two to six sections.[47] The most common isolates to cause URTA in cats and dogs are from the subgenus *Fumigati* section *Fumigati*, also known as the *A fumigatus* complex.[11,48–52] In contrast to dogs, in which *A fumigatus* is the single most common agent of SNA,[53] a more diverse range of *Aspergillus* species has been identified from cases of feline URTA (**Table 2**).[11,12,15,42]

Based on current evidence, *A fumigatus* (section *Fumigati*) and *A niger* (section *Nigri*) are the most common agents of SNA,[9,11,42] whereas a recently discovered species, *A felis* (section *Fumigati*), is the most common cause of SOA followed by *A udagawae* (section *Fumigati*) (see **Table 2**).[12,15,16] Section *Nigri* isolates, known as the black aspergilli, are phenotypically distinct from section *Fumigati* (**Fig. 1**).[54]

Recent Advances in Identification of Fungal Pathogens in URTA

The ability to accurately identify fungal species that cause aspergillosis has increased with the widespread availability of molecular techniques including polymerase chain reaction (PCR) and sequencing.[55] Members of the *A fumigatus* complex cannot be reliably identified on the basis of phenotypic features alone. *A fumigatus*–like or "cryptic" species have similar morphology and other phenotypic features to *A fumigatus*.[51] Misidentification of cryptic species causing feline URTA including

A felis, *A udagawae*, and *A lentulus* is likely when only morphologic typing methods are used.[11,12,51]

In four cases of SOA where *A fumigatus* was reported as the etiologic agent, isolates were identified by phenotypic features alone.[10,43–45] For three of the isolates where antifungal susceptibility to amphotericin B (AMB) was tested, minimum inhibitory concentrations of AMB were high, increasing the likelihood that these isolates were cryptic species.[10,43,44] Compared with *A fumigatus*, the minimum inhibitory concentrations of AMB for cryptic species, such as *A lentulus* and *A udagawae*, are high.[51,56] To date, of 35 cases of feline URTA in which the species identity of isolates was confirmed using PCR and sequencing of the internal transcribed spacer (ITS) and partial β-tubulin genes, *A fumigatus* has only been identified in cases of SNA (see **Table 2**) (Barrs and Talbot, unpublished data, 2013).[11,12,15,16]

Disseminated and Focal IA

The *Aspergillus* spp that cause disseminated IA and focal IA in cats remain largely unknown because most cases were diagnosed at postmortem only from histologic findings.[17–21] *A fumigatus* was identified from fungal culture morphology only in two cats with mycotic pneumonia,[25,28] one cat with mycotic cystitis,[26] and one cat with disseminated IA.[18] *A nidulans* was identified from fungal culture morphology only in one cat with mycotic cystitis.[27] Molecular confirmation of isolate identity was not performed in any case.

Current Fungal Taxonomy: What's in a Name?

The *A fumigatus* complex contains asexual members (anamorphs), many of which also have sexual forms (teleomorphs). The anamorph is typically mold-like and bears mitotic spores (conidia). The teleomorph is characterized by the production of meiotic spores (ascospores) that develop within sacs (asci) inside enclosed fruiting bodies (cleistothecia).[55]

Controversy has surrounded the fungal taxonomy of the *A fumigatus* complex because of the system of dual nomenclature used to describe anamorphic and teleomorphic phases of the same fungus. Traditionally the anamorphic phase was assigned to the genus *Aspergillus*, whereas the teleomorph of the same organism was assigned to the genus *Neosartorya*. The teleomorphic name received taxonomic precedence, such that species with known sexual stages were referred to by their teleomorph names.[57] Although this system of dual nomenclature provided a practical solution for distinguishing organisms that produce ascospores, confusion arose for such organisms as *A fumigatus*, where the teleomorph (*Neosartorya fumigata*) was only recently discovered and the taxon continued to be referred to by its anamorph name.[58]

In reforms to the *International Code of Nomenclature for Algae, Fungi and Plants* a "one-fungus, one-name" principle was adopted in July 2011.[59] In accordance with the Amsterdam declaration on fungal nomenclature, *Neosartorya* is now included in genus *Aspergillus* and teleomorphs are referred to by an informal cross-reference name (eg, *A fumigatus* [neosartorya-morph]).[60]

EPIDEMIOLOGY

Feline URTA occurs worldwide, with cases reported in Australia,[3,11–13,40] the United States,[7–10,41,42,45] Europe,[4,8,14,43] and Japan.[15,16] No age or gender predilection is apparent. The median age at diagnosis is 6.5 years (range, 16 mo to 13 years).[3–5,7,8,11,14,16,40–45]

Chapter 7

Table 1
Classification of respiratory aspergillosis in humans

Anatomic Location	Invasive/Noninvasive	Immune Status	Pathology
Lower respiratory tract	IPA (angioinvasive)	Immunocompromised • Prolonged severe neutropenia	Vascular invasion by fungal elements
	IPA (nonangioinvasive)	Immunocompromised nonneutropenic: • HIV/AIDS • Corticosteroids • Hematopoietic stem cell transplant recipients • Heritable immunologic defect (chronic granulomatous disease)	No evidence of vascular invasion Pyogranulomatous inflammatory infiltrate
	Chronic IPA (nonangioinvasive): • Chronic necrotizing PA • Chronic cavitary PA • Chronic fibrosing PA	Immunocompromised: • Structural lung disease (eg, neoplasia, asthma, emphysema, infection) • Corticosteroids • HIV/AIDS • Diabetes • Alcohol abuse	Hyphae mostly contained within cavity with only occasional direct tissue invasion (chronic necrotizing PA)
	Invasive bronchial aspergillosis	Immunocompromised	Hyphal invasion of large airways
	Aspergilloma • Noninvasive	Immunocompetent	Single cavity with fungal ball No hyphal invasion of parenchyma

Upper respiratory tract	Acute invasive FRS (angioinvasive)	Immunocompromised: • Neutropenia • Allogenic stem cell transplant recipients	Hyphal invasion of sinuses and contiguous structures (eg, orbit); coagulative necrosis, sparse inflammatory infiltrate, angioinvasion
	Chronic invasive FRS (angioinvasive)	Immunocompromised: • Diabetes • Corticosteroids • HIV/AIDS	Hyphal invasion of sinuses and contiguous structures (eg, orbit); infiltrative mass, mixed inflammatory response, angioinvasion
	Granulomatous FRS • Invasive	Immunocompetent: • Location-dependent disease (Sudan, Middle-East, Indian subcontinent)	Hyphal invasion of sinuses and contiguous structures (eg, orbit); highly cellular granulomatous inflammatory response, no angioinvasion; similar to feline sino-orbital aspergillosis
	Sinus aspergilloma (fungal ball) • Noninvasive	Immunocompetent: • Structural sinus disease (eg, dental root filling material)	Fungal mass within sinus; chronic nongranulomatous inflammatory response to fungal mass
	Allergic FRS • Noninvasive	Immunocompetent	An allergic/hypersensitivity response to the presence of extramucosal fungi within the sinus
	Chronic erosive noninvasive FRS	Immunocompetent	Similar to canine sinonasal aspergillosis; marked inflammatory response and sinonasal bony lysis

Abbreviations: FRS, fungal rhinosinusitis; IPA, invasive pulmonary aspergillosis; PA, pulmonary aspergillosis.

Table 2
Etiologic agents in genus *Aspergillus* of SNA and SOA in cats

Number of Cases		Identification (Phenotypic[P]/Molecular[M])		
SNA	SOA	Subgenus	Section	Species
7	0	*Fumigati*	*Fumigati*	*A fumigatus*[M]
1	0	*Fumigati*	*Fumigati*	*A lentulus*[M]
1	1	*Fumigati*	*Fumigati*	*N pseudofischeri* (*A thermomutatus*)[M]
1	18	*Fumigati*	*Fumigati*	*A felis*[M]
0	4	*Fumigati*	*Fumigati*	*A udagawae*[M]
0	1	*Fumigati*	*Fumigati*	*A virdinutans*[M]
3	0	*Circumdati*	*Nigri*	*A niger*[P (n=2), M (n=1)]

Molecular identification is based on polymerase chain reaction and sequencing of the internal transcribed spacer and β-tubulin regions.
Abbreviations: SNA, sinonasal aspergillosis; SOA, sino-orbital aspergillosis.
Data from Refs.[9,11,12,15,16,42]; and Barrs & Talbot unpublished data, 2013.

Of cases where serologic testing for feline immunodeficiency virus and feline leukemia virus was performed, only one cat tested positive for feline leukemia virus.[4] Diabetes mellitus, a recognized risk factor for aspergillosis in humans (see **Table 1**), was present in two cats diagnosed with URTA.[40,42] As has been reported in canine SNA,[39,61,62] feline URTA occurs occasionally in association with facial trauma, nasal neoplasia, and nasal foreign bodies (Barrs, unpublished data, 2013).

In contrast to canine SNA where dolicocephalic and mesaticephalic breeds are overrepresented,[39] pure-bred brachycephalic cats, predominantly of Persian or Himalayan breed, account for more than a third of all feline URTA cases.[3–5,7,8,11,14,16,40–45]

No gender or breed predisposition has been recorded for disseminated and focal IA in cats. Affected cats are usually young to middle-aged. Evidence of systemic immunocompromise in some cases included feline panleukopenia virus infection, feline leukemia virus infection, feline infectious peritonitis, or prolonged corticosteroid therapy.[17–21,30–32,46]

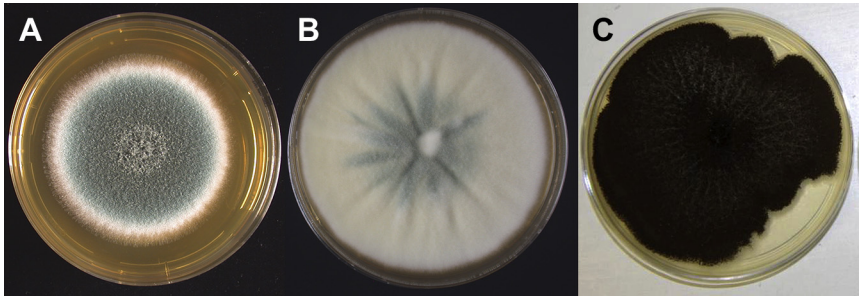

Fig. 1. Isolates of *Aspergillus fumigatus* (*A*), *Aspergillus felis* (*B*), and *Aspergillus niger* (*C*) on malt extract agar, from cats with URT aspergillosis. The black aspergilli (Section *Nigri*; [*C*]) are phenotypically distinct from Section *Fumigati* (*A*, *B*). Species within Section *Fumigati* cannot be reliably identified from phenotypic features. Some, like *A felis* (*B*), are generally slow to sporulate.

PATHOGENESIS

The ability of fungi to cause disease depends on a complex interplay between the pathogen (virulence factors) and the host (innate and adaptive immune responses). In humans, host factors that predispose individuals to IA include disorders of innate immunity, such as reduced mucociliary clearance (eg, cystic fibrosis); decreased numbers of phagocytic cells (ie, neutropenia); and phagocytic cell dysfunction (eg, chronic granulomatous disease, in which there is impaired production of oxidative intermediates).[63]

After inhalation, *Aspergillus* conidia that escape mucociliary clearance are mostly phagocytosed by macrophages and dendritic cells.[34] Phagocytic host cells express pattern recognition receptors (PRRs) that recognize specific fungal epitopes known as pathogen-associated molecular patterns (PAMPs) and damaged host cell components known as damage-associated molecular patterns (DAMPs). The major PAMPs of filamentous fungi are cell-wall components including β-glucans, chitin, and mannans, whereas DAMPs include nucleic acids and alarmins. The major PRRs of host cells, as characterized in humans, include C-type lectin receptors; toll-like receptors (TLRs); nucleotide oligomerization domain-like receptors (NOD-like receptors or NLRs); and galectin family proteins.[63,64]

Is URTA Associated with a Disorder of Innate Immunity?

To date no studies have been performed to investigate innate or immune responses in cats with URTA. Several single nucleotide polymorphisms in PRRs that increase susceptibility to IA have been described in humans including single nucleotide polymorphisms in TLRs 1, 3, 4, and 6, and in the C-type lectin receptors Dectin-1 and DC-SIGN.[64,65] Whether similar genetic mutations could be associated with increased susceptibility to URTA in Persian/Himalayan cats has not been investigated. By contrast, several studies have evaluated the immune response to SNA in dogs.[66–69] It is characterized by a dominant T-helper cell 1 (Th1) response with upregulation of interleukin-10. A dominant Th1 response correlates with protective antifungal immunity and is thought to be important in preventing invasive disease.[39] Upregulation of interleukin-10 is important in limiting the extent of local tissue destruction[70] but paradoxically could also be the reason why affected dogs are unable to clear infection spontaneously. The inflammatory process in fungal infection is beneficial in containing the infection but an uncontrolled inflammatory response is detrimental and might inhibit disease eradication.[64]

To explore the hypothesis that a dysfunction in innate immunity could be an etiologic factor in the development of canine SNA, the expression of messenger RNA (mRNA) encoding TLRs 1 to 10 and NLRs 1 and 2 was quantified in nasal mucosal biopsies from dogs with SNA and control dogs using quantitative real-time PCR.[68] In dogs with SNA there was significantly higher expression of all PRRs except for TLR3, TLR5, and NLR1 compared with normal dogs. The significance of these findings is unknown because little is known about the function of PRRs in canine nasal immunity in health and disease.

Is URTA Associated with Impaired Mucociliary Clearance?

The increased risk of URTA observed in brachycephalic cats could reflect reduced mucociliary clearance from abnormal sinonasal cavity conformation. Decreased sinus aeration and drainage of respiratory secretions secondary to infection, polyps, and allergic rhinosinusitis is a risk factor for invasive fungal rhinosinusitis in humans.[71] Mucosal edema and impaired drainage of URT secretions caused by turbulent airflow

and abnormal facial conformation has been proposed as a risk factor for fungal colonization in brachycephalic cats.[8] However, because brachycephalic dogs are underrepresented for SNA, additional risk factors, such as previous viral URT infection or recurrent antimicrobial therapy, could also be involved.[4,8,11]

Fungal Virulence Factors

An important virulence factor of *Aspergillus* spp is their thermotolerant nature that enables survival in mammals.[72] Toxic secondary metabolites are associated with host immunosuppression or evasion of the immune system.[73] Gliotoxin, a mycelial-derived product, prevents phagocytosis by macrophages, reduces T-cell proliferation and activation, and induces macrophage apoptosis.[73–75] Other putative fungal virulence factors include the ability to adhere to host tissue by conidia and laminin-binding components, factors interfering with fungal cell opsonization, and the production of proteases capable of macromolecule degradation to provide fungal nutrients.[73] Species-specific fungal virulence factors could be involved in the development of invasive URTA because different fungal species are implicated in SNA and SOA in cats.[12]

Progression from SNA to SOA

SOA results from extension of a primary sinonasal infection to involve paranasal structures including, but not limited to the orbit. The evidence for this includes:

- Documented progression of disease from SNA to SOA.[7]
- History of nasal signs preceding development of an orbital fungal granuloma.[3,7,11,16,45]
- Detection of concurrent sinonasal cavity involvement on imaging, at surgery, or at necropsy.[3,6,10,11]
- Detection of a direct communication between the orbit and nasal cavity on computed tomography, surgery, or necropsy in the orbital lamina and less commonly the frontal bone.[11,43,76]
- Isolation of *A felis*, the most common cause of SOA, from a cat with SNA.[12] A large defect in the orbital lamina was present on computed tomography. Infection was arrested with aggressive antifungal therapy and SOA did not develop.[11]

CLINICAL PRESENTATION

Clinical findings in feline SNA (outlined in **Box 1**) are similar to those reported for chronic rhinosinusitis (discussed elsewhere in this issue). Most cats with SOA are presented for clinical signs associated with an invasive retrobulbar fungal granuloma (**Box 2**, **Fig. 2**).[3,7,10,11,14–16,43–45] In most cats exophthalmos is unilateral, but in severe, chronic infections, bilateral exophthalmos can occur.[3,11,43] Nasal signs are absent in 40% of SOA cases at presentation; however, the medical history usually reveals sneezing or nasal discharge in the preceding 6 months. Pain on opening the mouth and neurologic signs are uncommon at initial presentation. However, cats with advanced disease are often euthanized because of the development of neurologic signs, which can include seizures, nystagmus, circling, facial muscle fasciculation, hyperesthesia, and blindness.[11,44,45]

DIFFERENTIAL DIAGNOSES

Differential diagnoses for cats presenting with chronic nasal signs are listed in **Box 3** and for cats presenting with exophthalmos are listed in **Box 4**. Brachycephalic

Box 1
Clinical signs in sinonasal aspergillosis

Common signs

- Sneezing
- Stertor
- Unilateral or bilateral serous to mucopurulent nasal discharge
- Ipsilateral mild mandibular lymphadenopathy

Less common signs

- Epistaxis (30% of cases)
- Fever
- Discharging sinus or soft tissue mass involving the nasal bone or frontal sinus

conformation should increase suspicion for aspergillosis, although these cats are also overrepresented for viral URT infections. Where epistaxis is present neoplasia, mycotic rhinitis, or severe chronic rhinosinusitis are more likely, along with systemic hypertension. Inability to retropulse the globe and measurement of intraocular pressure enables differentiation of exophthalmos from buphthalmos (abnormal enlargement of the globe).[45] Other infectious or neoplastic processes extending from the nasal cavity to the orbit can have a similar presentation to SOA, including cryptococcosis, nasal lymphoma, and nasal carcinoma.

Box 2
Clinical signs in sino-orbital aspergillosis

Common signs

- Nasal signs (clinical or historical finding within the previous 6 months)
- Unilateral exophthalmos with dorsolateral deviation of the globe
- Ipsilateral conjunctival hyperemia
- Ipsilateral prolapse of the nictitating membrane
- Ipsilateral exposure keratitis
- Oral mass or ulcer in the ipsilateral pterygopalatine fossa
- Paranasal soft tissue swelling
- Nasal signs
- Mild mandibular lymphadenopathy

Less common signs

- Fever
- Bilateral exophthalmos
- Ulceration of the hard palate
- Neurologic signs
- Discharging sinus (facial)

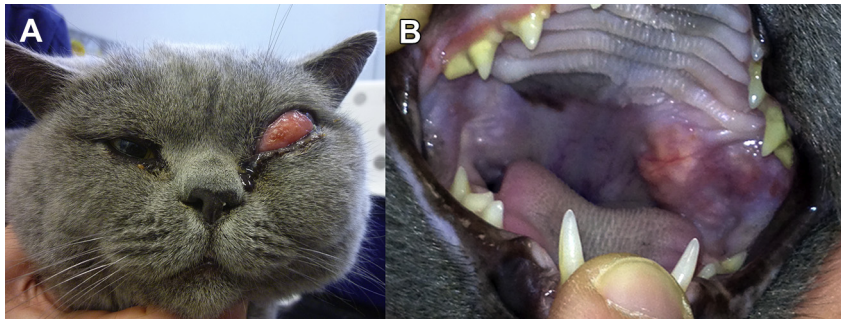

Fig. 2. British Shorthair cat with sino-orbital aspergillosis. Infection was caused by *Aspergillus felis*. Note the left-sided third eyelid prolapse, hyperemia, and edema, and the paranasal soft tissue swelling (*A*), and the pterygopalatine fossa mass (*B*), caused by an invasive retrobulbar fungal granuloma.

DIAGNOSIS

Diagnosis of feline URTA requires various combinations of serology, advanced imaging, rhinosinuscopy, cytology, histology, fungal culture, and molecular identification. Definitive diagnosis is based on identification of fungal hyphae on cytologic or histologic examination of tissue biopsies or sinonasal fungal plaques. Similar to canine SNA, diagnosis can also be made by visualization of sinonasal fungal plaques on endoscopy.[4,42] However, given the more diverse range of fungal pathogens that can cause feline URTA, definitive identification of fungal pathogens should always be attempted.

Hematology and Biochemistry

Hematology is unremarkable or there is evidence of a stress or inflammatory leukogram. Peripheral eosinophilia is uncommon (10% of cases).[7,8,10,11,16,42–45] Mild to severe hyperglobulinemia is the most common abnormality on serum biochemistry. This was reported in 9 of 16 cats with SOA and one cat with SNA caused by *A felis* infection.[7,10,11,45] This finding suggests that in cats with confirmed URTA the presence of hyperglobulinemia is a marker for invasive disease. However, prospective studies are required to investigate this.

Box 3
Differential diagnoses of nasal signs

- Neoplasia (lymphoma, carcinoma, other)
- Inflammatory (chronic rhinosinusitis, nasal/nasopharyngeal polyp, nasopharyngeal stenosis)
- Infectious
  - Viral (Feline Herpesvirus-1, Feline Calicivirus)
  - Mycotic rhinitis (cryptococcosis, aspergillosis, sporotrichosis, phaeohyphomycoses, other)
  - Bacterial (*Bordetella*, *Mycoplasma*, *Chlamydophila felis*, *Actinomycetes*)
- Foreign body
- Congenital (choanal atresia, palatine defects)
- Dental disease (oronasal fistula)

Box 4
Differential diagnoses of orbital mass lesions

- Neoplasia
  - Lymphoma
  - Adenocarcinoma/undifferentiated carcinoma
  - Squamous cell carcinoma
  - Fibrosarcoma
  - Osteoma/osteosarcoma
  - Other
- Infectious
  - Bacterial abscess/granuloma (odontogenic, penetrating bite wound, hematogenous)
  - Mycotic granuloma
    - Aspergillosis
    - Cryptococcosis
    - Penicilliosis
    - Phaeohyphomycosis
    - Hyalohyphomycosis
  - Pythiosis
- Inflammatory
  - Orbital myofascitis
  - Orbital pseudotumor (idiopathic sclerosing inflammation)
  - Zygomatic or lacrimal adenitis
- Foreign body (eg, grass awn)
- Orbital fat prolapse

Serology

Aspergillus antigen detection in URTA

Galactomannan (GM) is a polysaccharide component of the cell wall of *Aspergillus* and other filamentous fungal species that is released into the circulation during hyphal invasion into tissue.[77] A recent study evaluated serum GM measurement for diagnosis of feline URTA.[78] A one-stage, immunoenzymatic sandwich enzyme-linked immunosorbent assay (ELISA) (Platelia, Bio-Rad, Marnes-la-Coquette, France) was used to detect serum GM in four groups of cats: Group 1 cats had confirmed URTA (N = 13; six SNA, seven SOA); Group 2 cats had other URT diseases (N = 15); Group 3 cats were treated with β-lactam antibiotics for non–respiratory tract disease (N = 14); and Group 4 were healthy cats (Group 4a cats $\leq$1 year of age, N = 28; Group 4b cats >1 year of age, N = 16). Using a cut-off optical density index of 1.5, 3 of 13 cats with URTA (two SOA, one SNA) tested positive for serum GM. The overall sensitivity and specificity of the assay was 23% and 78%, respectively. False-positive results occurred in 29% of cats in Group 3 and 32% of cats in Group 4a. Specificity increased to 90% when Groups 3 and 4a were excluded from the analysis.

The low sensitivity of detection of serum GM in feline URTA is likely to be associated with systemic immunocompetence of the host. In humans, the sensitivity of the

GM ELISA for detection of IA is more than 90% in neutropenic patients,[79] whereas in nonneutropenic patients the sensitivity is less than 30%.[80,81] In the former, antigen is cleared by neutrophils, which possess mannose-binding receptors, or by complexing with circulating anti-*Aspergillus* antibodies.[82,83] Also, low sensitivity of GM detection in feline SNA could reflect absence of tissue invasion, as is the case in canine SNA where fungal hyphae colonize superficially but do not penetrate the sinonasal epithelium.[84]

The poor specificity of the Platelia GM assay for diagnosis of feline URTA mimics the situation in humans where false-positive results have been identified in pediatric patients, and in patients treated with β-lactam antibiotics that contain small amounts of GM introduced during the manufacturing process.[85,86] Except in the setting of ruling out URTA in cats with respiratory disease, serum GM is not useful as a routine diagnostic test for feline URTA.

Antibody tests

Serum anti-*Aspergillus* antibodies can be detected by numerous methods including counter-immunoelectrophoresis, agar gel immunodiffusion, or ELISA. Results of serologic tests performed in commercial laboratories have been published in case-reports totaling 10 cats with URTA (nine SNA, one SOA), of which five were seropositive.[4,8,9,11,42]

Preliminary results of a prospective study to evaluate the diagnostic sensitivity and specificity of anti-*Aspergillus* antibody detection in feline URTA indicate that antibodies to *A felis* cross-react with the same aspergillin preparation evaluated for diagnosis of canine SNA (Aspergillus Immunodiffusion Antigen, Meridian Bioscience, Cincinnati, Ohio, USA) (Barrs, unpublished data, 2013).

Diagnostic Imaging: Computed Tomography and Magnetic Resonance Imaging

Advanced imaging (computed tomography [CT] or magnetic resonance imaging [MRI]) is recommended for all cases of suspected feline URTA. As for canine SNA, cribriform plate integrity should be assessed before treatment with topical antifungal preparations. Evidence of invasive disease, including paranasal soft tissue infiltration and orbital involvement, may not be apparent on physical examination. Determination of orbital involvement will affect subsequent case management.[11] CT is generally superior to MRI for evaluation of destructive lesions in bony structures contiguous with the sinonasal cavity. In cats with suspected intracranial extension of infection, MRI after intravenous contrast administration is superior to CT for evaluation of intracranial soft tissues.

CT findings in SNA

CT findings in cats with SNA have been reported in a small number of cases and seem to be more variable than in canine SNA.[9,41,42,76] Common findings are listed in **Box 5**. In one study of five cats with SNA, findings that are relatively specific for SNA in dogs, including cavitated-like turbinate lysis, mucosal rim thickening adjacent to the bones of the sinonasal cavity, soft tissue accumulation, and reactive bony-changes, were absent.[41] However, these changes have been observed recently in cats with SNA caused by *A fumigatus* (Barrs, unpublished data, 2013).

A calcified nasal cavity concretion was reported in one case of feline SNA.[8] Calcification of fungal plaques occurs in sinonasal *Aspergillus* spp infections in humans, in approximately 50% of non-IA sinus fungal balls with maxillary sinus involvement.[87] It is caused by deposition of calcium oxalate or phosphate crystals that are thought to be fungal metabolites.

Box 5
CT findings in SNA

- Nasal cavity involvement is usually bilateral
- Turbinate lysis
- Increased soft tissue attenuation within the nasal cavities
- Fluid or soft tissue accumulation within frontal and sphenoid sinuses

Additional CT findings in SOA

- Ventromedial orbital mass
- Dorsolateral displacement of the globe, which may be indented
- Heterogeneous and peripheral rim post contrast enhancement of orbital mass
- Paranasal soft tissue mass effect: pterygopalatine fossa, adjacent maxilla
- Lytic lesions in paranasal bones

CT findings in SOA

CT Features of SOA (see **Box 5**) overlap those seen in malignant nasal neoplasia (eg, nasal lymphoma, nasal carcinomas) and other invasive mycoses, such as cryptococcosis (**Fig. 3**).[41] Overlapping features include osteolysis of paranasal bones, moderate to severe turbinate destruction, mass-effect, and extension of disease into the orbit or paranasal soft tissues.[43–45,76,88,89]

Biopsy Procedures

Endoscopic visualization of the sinonasal cavity can be performed using nasopharyngoscopy, rhinoscopy, and sinuscopy. Biopsy specimens are obtained for cytology and/or histology, and culture. Biopsy specimens can be stored frozen for PCR if URTA is suspected but fungal culture is negative. Nasal cavity lavage may yield larger biopsy specimens than can be acquired endoscopically and assists in debridement of mucosal plaques (**Fig. 4**).

Sinuscopy is indicated when CT findings indicate sinus involvement and fungal plaques are not visualized on rhinoscopy. Anatomic landmarks have been defined for sinus trephination in cats.[90] Trephine openings are made slightly to the side of the mid-line on a line that joins the anterior borders of the supraorbital processes.

For cats with SOA, biopsies of retrobulbar masses can be obtained by the oral cavity where there is pterygopalatine invasion.[11] CT-guided biopsies of orbital or other paranasal mass lesions can be performed.[45]

Fungal culture

Fungal pathogens that cause feline URTA can be readily cultured from tissue biopsies or fungal plaques using commercial culture media. In one study 22 of 23 cases of feline URTA were culture positive.[11] Culture of nasal swabs for diagnosis of feline URTA has not been evaluated.

Molecular identification of fungi

In the clinical setting comparative DNA sequence analysis used in conjunction with traditional phenotype-based methods is a practical approach to identification of species within the genus *Aspergillus*.[52] Fungal DNA can be extracted for sequencing directly from fresh or frozen clinical specimens, or from fungal culture material. One

Chapter 7

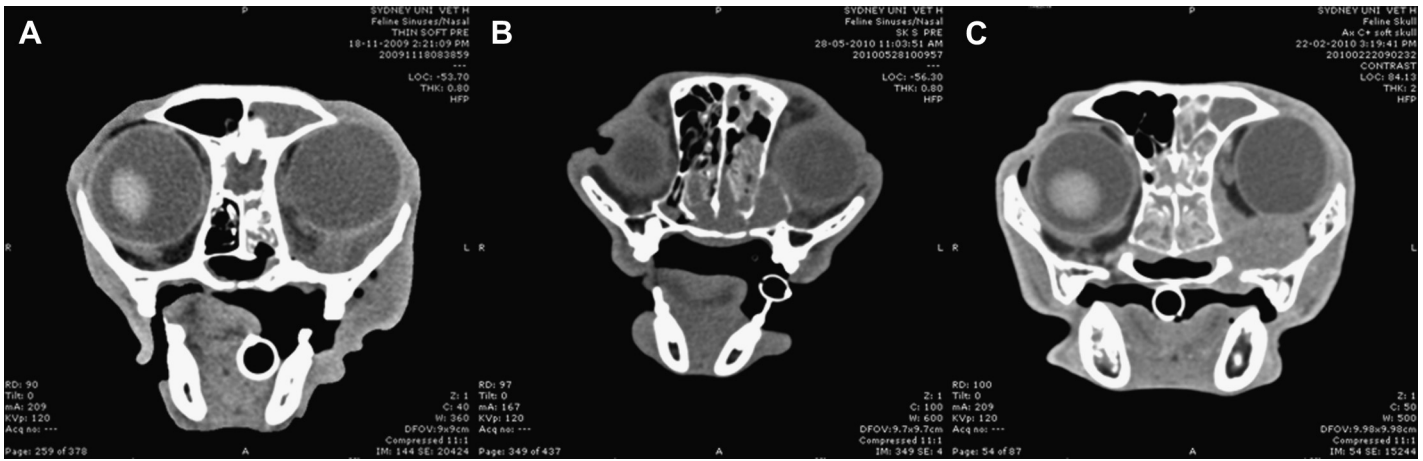

Fig. 3. CT features of sino-orbital aspergillosis (*A*), including nasal cavity soft tissue attenuation, lysis of paranasal bones, and mass-effect (ventromedial orbital mass), overlap those of other mycoses (eg, cryptococcosis) (*B*), and neoplasia (eg, lymphoma) (*C*).

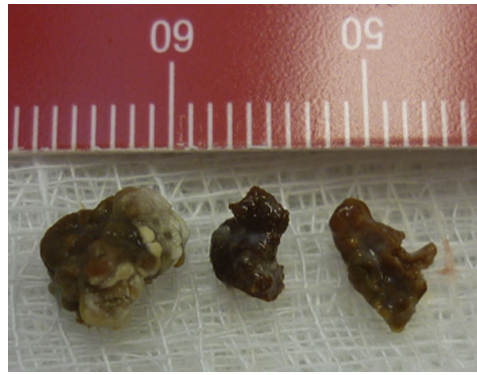

Fig. 4. Plaques of *Aspergillus fumigatus* retrieved from the nasopharynx of a cat after nasal lavage under general anesthesia, with sterile saline.

of the limitations of using formalin-fixed paraffin embedded tissues is that they often only yield short DNA fragments, thus limiting the gene targets for amplification.

The genome of all fungi contains multiple copies of the ribosomal DNA (rDNA) gene complex, consisting of highly variable regions, the ITS regions, which are flanked by highly conserved gene sequences that are suitable targets for primers. Sequence heterogeneity within the ITS regions is useful for the separation of genera and species, and appropriately exploited as a "panfungal" PCR for identification of fungi in clinical specimens.[91,92] The rDNA gene complex includes three genes: (1) the 18s rDNA gene, also known as the small-subunit rDNA gene, which is 1800 base pairs (bp) long; (2) the 5.8S gene (159 bp); and (3) the 28S rDNA, also known as the large-subunit rDNA gene (3396 bp) (**Fig. 5**).[93] Comparative sequence analysis of ITS1-5.8S-ITS2 is an appropriate locus to first identify *Aspergillus* isolates to the level of subgenus/complex.[52] However, because some closely related species show little or no variation in ITS sequences, accurate identification of the fungal species requires additional analyses of one or more partial gene regions.[94]

Histopathology

SNA

Histologic changes in canine SNA are characterized by ulcerated and severely inflamed mucosa, often covered by a plaque of necrotic tissue containing hyphae, and/or luminal exudates containing hyphae.[9,42,84] Fungal hyphae do not penetrate the mucosal epithelium, and the underlying lamina propria is typically heavily infiltrated by a dense sheet of mixed mononuclear inflammatory cells. Histologic changes in noninvasive feline SNA have not been reported systematically but seem similar to canine SNA. Changes include severe inflammatory rhinitis with lymphoplasmacytic

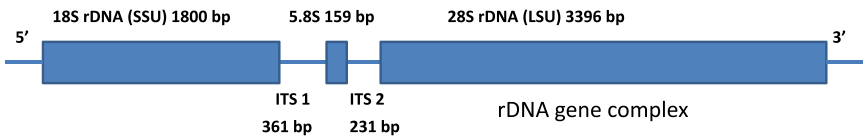

Fig. 5. Representation of the ribosomal DNA (rDNA) gene complex in fungi denoting gene order of small subunit (SSU) and large subunit (LSU) and position of internal transcribed spacer (ITS) regions.

or mixed-cell inflammatory cell infiltrates; necrosis, which can be extensive; and mats of fungal hyphae.[8,9,11,42] Histopathologic detection of tissue invasion by fungal hyphae is important because progression to SOA can occur in invasive infections, and evidence of invasion affects treatment decisions (discussed later).[8]

SOA

Cats with SOA have granulomatous invasive mycotic rhinitis and sinusitis with variable submucosal invasion and bony lysis.[3,11,43] Granulomas are comprised of central necrotic cellular debris within which parallel-walled dichotomously branching, septate fungal hyphae are confined that can be readily identified using special stains (eg, periodic acid–Schiff, Grocott methenamine silver, or Gridley stain).[3,7,10,11,43,45] Surrounding the central area of coagulative necrosis are zones of inflammatory cells and peripheral fibrosis that wall off the hyphae. In some lesions eosinophilic inflammation is prolific, whereas in others neutrophilic inflammation is predominant. Adjacent to these are activated and epithelioid macrophages with vacuolated cytoplasm that form sheets in some places. Peripherally there is a zone of fibroblasts and a cuff of lymphocytes and plasma cells. Inflammatory lesions can efface adjacent skeletal muscle and bone.[11] The globe is resistant to infiltration by fungal hyphae; however, invasion of adjacent structures including the nictitating membrane and eyelid has been observed (Barrs and Day MJ, unpublished data, 2011).[10,44] Mycotic invasion of the optic nerve and optic chiasm in cats that developed blindness has been reported.[11] Development of central nervous system signs in some cases implies fungal invasion of central nervous system tissue; however, brain histology was not performed in the two cats in which seizures were described.[44,45]

TREATMENT AND PROGNOSIS

SNA

The prognosis for feline SNA is favorable, although only small numbers of cases have been treated. Signs resolved in 11 of 14 treated cases in which follow-up information was available.[4,8,9,11,42] Successful treatment regimes included systemic antifungal therapy alone in five cases (itraconazole or posaconazole monotherapy or combined with AMB); systemic triazole therapy (itraconazole or posaconazole) and topical intranasal clotrimazole infusion in two cases; and topical intranasal clotrimazole infusion alone in two cases. As for canine SNA,[95] debridement of fungal lesions in the nasal cavity was an important part of therapy for most cases.[4,8,11,42]

Evidence-based treatment protocols are not available. A suggested therapeutic strategy for treatment of feline SNA, based on previous reports, treatment of canine SNA, and considering whether infection is invasive or noninvasive, is outlined in **Box 6**. Because of the propensity for feline SNA to progress to SOA, where there is any doubt about the presence of invasion, or where *A felis*, a species highly correlated with invasive disease, is identified, concurrent topical and systemic therapy is recommended.

Techniques for intranasal clotrimazole infusion are adapted from procedures used to treat canine SNA and are described in detail elsewhere.[8,39,42] Polyethylene glycol should be used as the vehicle for 1% intranasal clotrimazole infusions, not polypropylene glycol, because the latter can cause severe mucosal edema and ulceration.[96] Response to therapy can be assessed from repeat CT and endoscopy. As for canine SNA, multiple clotrimazole infusions may be required to resolve infection.[11]

SOA

SOA carries a poor prognosis. Optimal treatment protocols for treatment of SOA have not been identified. Based on treatment responses in individual cases[7,10,11,16,45] the

Box 6
Therapeutic approach for treatment of feline SNA

- Record the identity of the fungal isolate and its antifungal susceptibility.
- Assess whether infection is invasive or noninvasive based on histopathology and CT findings.
- Determine the integrity of the cribriform plate on CT.
- Debride fungal plaques/lesions from the nasal cavity and frontal sinuses using endoscopic techniques and saline irrigation.
- For noninvasive infections instill an intranasal infusion of 1% clotrimazole in polyethylene glycol (1 hour soak under general anesthesia). Ensure drainage of the infusion from the nasal cavities at the end of the procedure by tilting the head.
- For invasive infections or where *Aspergillus felis* is identified, give additional systemic antifungal therapy (see SOA treatment).

recommended therapeutic approach is posaconazole or itraconazole given as monotherapy or combined with AMB (**Table 3**). Administration of systemic antifungals for 6 months or longer can be necessary in some cases and reinfection or relapse of infection can occur.[11,16] *A felis*, the most common cause of SOA, usually shows in vitro susceptibility to AMB.[12] AMB was used in the successful treatment of one case of SNA and one case of SOA caused by *A felis*, in combination with itraconazole or posaconazole.[11]

Surgical debridement of large granulomas is logical but a clear advantage over medical therapy has not been demonstrated in SOA. Three of six cases that responded to therapy had surgery including orbital exenteration in two cases and debridement of the orbital granuloma in the other.[7,10,45] One of these also had lavage of orbital tissues at surgery with 1% voriconazole.[45] In only one of these cases was resolution of infection confirmed by follow-up CT[10] and one cat was euthanized 4 months after exenteration, with likely progressive disease.[7] In the largest case series of 12 cats with SOA for which treatment outcomes could be assessed, there were 11 treatment failures including five cases treated with combined surgery and antifungal therapy and six with medical therapy alone. One case treated successfully with medical therapy alone relapsed 19 months after treatment was stopped, responded to further medical therapy, and is disease free 5 years later.[11]

Fluconazole is not recommended for treatment of aspergillosis because most *Aspergillus* species are resistant to this drug. Antifungal susceptibility testing should be performed before treatment, because resistance to AMB and some triazole drugs has been identified in isolates from cases of SOA.[10,12,43,44]

Establishing pretreatment renal function and base-line liver enzymes is also important because nephrotoxicity and hepatotoxicity is common with some antifungal drugs (see **Table 3**).

Voriconazole (structurally similar to fluconazole) and posaconazole (structurally similar to itraconazole) are fungicidal triazoles that were developed as more efficacious agents for treatment and prophylaxis of IA in humans and to improve on the absorption, tolerability, and drug interaction profile of itraconazole. Although their pharmacologic characteristics have not been determined in cats, posaconazole is well tolerated after oral administration and liver enzyme elevations are infrequent.[10,11,44] Serious adverse neurologic effects (hindlimb paraplegia and blindness) were reported after voriconazole administration in cats,[11,45,97] and it is not recommended for treatment of feline URTA unless other therapies have failed.

Table 3
Dosages of antifungals used in the treatment of feline upper respiratory tract aspergillosis

Drug/Formulation	Dosage/Route of Administration	Adverse Effects
Itraconazole 100-mg capsules 10 mg/mL oral suspension (Sporanox)	Capsules: 5 mg/kg q 12 h or 10 mg/kg q 24 h PO Administer with food Oral suspension: 1–1.5 mg/kg q 24 h PO	Gastrointestinal: anorexia, vomiting Hepatotoxicity: elevated liver enzyme levels, jaundice. Monitor ALP/ALT monthly. If hepatotoxicity occurs, reduce dosage to 5 mg/kg q 24 h or 10 mg/kg q 48 h PO (capsules)
Posaconazole 40 mg/mL liquid (Noxafil)	5–7.5 mg/kg divided twice daily PO Administer with food	Hepatotoxicity: unlikely to occur at 5 mg/kg divided twice daily PO
Voriconazole 50-mg tablets 40 mg/mL powder for oral suspension (Vfend)	5 mg/kg q 24 h PO	Gastrointestinal: anorexia Neurologic: blindness, ataxia, stupor, hind-limb paraplegia. Consider use only when other therapies have failed
Terbinafine 250-mg tablets (Lamisil)	30 mg/kg q 24 h PO	Gastrointestinal: anorexia, vomiting, diarrhea
Amphotericin B deoxycholate 50-mg vial (Fungizone)	0.5 mg/kg of 5 mg/mL stock solution in 350 mL per cat of 0.45% NaCl + 2.5% dextrose SC two or three times weekly to a cumulative dose of 10–15 mg/kg	Nephrotoxicity: monitor urea/creatinine every 2 wk. Discontinue for 2–3 wk if azotemic
Liposomal amphotericin (AmBisome)	1–1.5 mg/kg IV q 48 h to a cumulative dose of 12–15 mg/kg Give as a 1–2 mg/mL solution in 5% dextrose by IV infusion over 1–2 h	Nephrotoxicity: less nephrotoxic than amphotericin B, but azotemia can occur. Monitor urea/creatinine 1–2 × weekly

Echinocandins, a novel class of semisynthetic amphiphilic lipopetides, inhibit synthesis of the fungal cell wall component 1,3-β-glucan. They are used for treatment of refractory IA in humans.[98] Caspofungin was well tolerated and efficacious in one cat with SOA that failed treatment with AMB and posaconazole.[11] In another case treatment with micafungin was unsuccessful.[15] As with other polypeptides, echinocandins can cause histamine release.

SUMMARY

Feline URTA, the most commonly reported form of aspergillosis in cats, commences as an infection in the nasal cavity. In SNA, infections remain confined to the sinonasal cavity. In SOA, which is an invasive mycosis, infection extends from the nasal cavity to involve paranasal structures, including the orbit. In contrast to canine SNA, feline URTA is caused by a diverse range of *Aspergillus* species, mostly from the *A fumigatus* complex. Phenotypic methods of identification are unreliable, but fungi can be readily identified using PCR and sequencing of the ITS and

β-tubulin gene regions. SNA carries a favorable prognosis with treatment, whereas the prognosis for SOA is poor. Optimal treatment regimes for feline URTA have not been identified.

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7.4 Further discussion

Further characterisation of the *Aspergillus viridinutans* complex

Before I commenced my research, the *A. viridinutans* complex was known to consist of three species: *A. viridinutans, A. aureolus* and *A. udagawae*. *A. viridinutans* was first isolated from sand and rabbit dung in Frankston, near the Mornington Peninsula in Victoria in 1954 (McLennan et al., 1954). Interestingly, although *A. viridinutans* sensu stricto was the first to be discovered in the *A. viridinutans complex,* it is also the least common isolate in the *A. viridinutans* complex. In fact, astonishingly, no other isolate identical to the type-strain described by McLennan and others (1954), based on molecular methods, has been published. *A. viridinutans* can grow at 42 ^{o}C but not at 45 ^{o}C (Barrs et al., 2013a).

A. aureolus was first characterised in 1955 by Fennell and Raper. The two type strains were identified from soil isolated during the spring of 1950s from samples of soil submitted by C.F. Charter, West African Cacao Research Institute, Tafo, Gold Coast, Africa and by Prof. J.T. Baldwin, Monrovia, Liberia, respectively. It has also been isolated from soil in other tropical countries including Brazil, Ghana and Suriname, and from passion-fruit juice in Fiji (Novakova et al., 2013). *A. aureolus* is the only homothallic species within the complex, and ascomata are visible within one week of incubation on malt extract agar (MEA) at 25 ^{o}C (Novakova et al., 2013). A pathogenic role for *A. aureolus* is yet to be ascribed. *A. aureolus* can grow at 42 ^{o}C but not at 45 ^{o}C.

A. udagawae was first characterised in 1995 from an isolate from Brazilian soil (Horie et al., 1995). Since then it has also been isolated from soil in other parts of South America, from Korea and the USA (Novakova et al., 2013). It was the first heterothallic species with a fully functioning reproductive cycle to be described in the *A. viridinutans* complex. Ascomata are visible after 3 weeks incubation on MEA at 25 ^{o}C.

With the advent of molecular methods of identification, *A. udagawae* has been identified as an emerging agent of aspergillosis in humans, as previously discussed. It has been

isolated from human lung, upper respiratory tract, brain, cornea and nail from the Czech Republic, Italy, Japan and the USA (Balajee et al., 2006; Gyotoku et al., 2012; Posteraro et al., 2011; Sugui et al., 2010; Vinh et al., 2009b; Yaguchi et al., 2007). My research and that of others has established *A. udagawae* as the second most common cause of SOA in cats (Barrs et al., 2014; Barrs et al., 2013a; Kano et al., 2008; Kano et al., 2013).

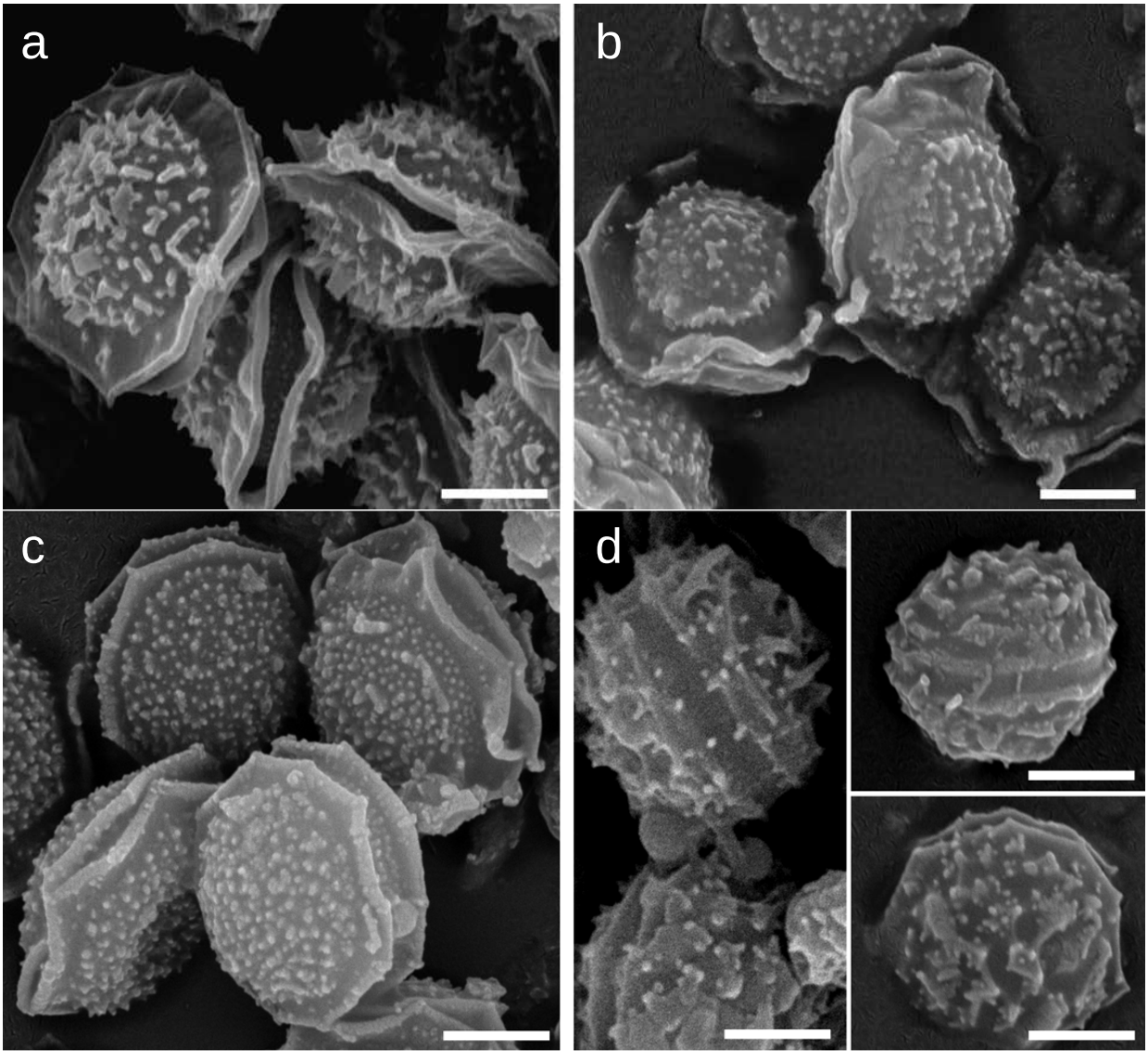
In 2013 I discovered a fourth member of the *A. viridinutans* complex, namely *A. felis*, and demonstrated that it is a heterothallic species with a fully functional reproductive cycle (Barrs et al., 2013a). Slightly later in 2013, Novakova and others discovered a fifth member of the *A. viridinutans* complex, *A. wyomingensis*, from a soil reclamation site in Wyoming, USA. In naming *A. wyomingensis,* the research team adhered to the "one fungus one name principle" that we adopted as per the revisions of the International Code of Botanical Nomenclature discussed in Chapter 2. Support for these nomenclature revisions seems to be building amongst mycologists.

A. wyomingensis is also heterothallic and has a functional sexual cycle. Maximum growth temperature studies were performed for all *A. viridinutans* complex members. I have since discovered an isolate of *A. wyomingensis* in a cat with SOA and am the first to ascribe a pathogenic role to this species.

Another finding in the study by Novokova and others (2013) was that all isolated species had maximum growth temperature of between 42 and 45 ^{o}C. All isolates grew at 42 ^{o}C and none grew at 47 ^{o}C. Similar to the findings of my study, *A. viridinutans* had a maximum growth temperature of 42 ^{o}C and did not grow at 45 ^{o}C. In contrast to my study where all isolates of *A. felis* grew restrictedly at 45 ^{o}C, Novakova found growth at 45 ^{o}C was isolate-dependent for *A. felis* and also for *A. wyomingensis* and *A. udagawae.* The morphology of ascospores alone was found to clearly differentiate all heterothallic species within the *A. viridinutans* complex (see panels D and E in Figure 4 of Barrs and others 2013, Chapter 4). This is in marked contrast to anamorph morphology, which is notoriously unreliable in *Aspergillus* section *Fumigati* taxonomy.

Interestingly, however, when *A. viridinutans* was first discovered, the feature of “nodding heads” was described, where the arrangement of the vesicle and phialides is at such angle as to present a “nodding appearance” (McLennan et al., 1954). Although upright heads were often seen, the nodding type was more frequent. Put more simply, some vesicles are borne at an angle to the stipe. I found that *A. felis* had this similar feature. This can be seen in panels D and E from the monograph of *A. felis* that I published in 2013 (**Figure 7.4.2**). Similarly, Novokova found that nodding heads nodding heads occurred in all species within the *A. viridinutans* complex. However, the feature is not reliable for morphological identification because it is also present in some non-related species from *Aspergillus* section *Fumigati* including *A. brevipes, A. duricaulis, .A marvanovae*, *A. unilateralis, N. pseudofischeri (A. thermomutatus*), *A. brevistipitatus* and *A. conversis* (McLennan et al., 1954; Novakova et al., 2013; Smith, 1952; Varga et al., 2000)

Figure 7.1.4 Comparison of ascospore morphology of homothallic *A. aureolus* and three heterothallic species in *A. viridinutans* complex as observed by scanning electron microscopy a) *A. aureolus*, b) *A. felis,* c) *A. udagawae,* d) *A. wyomingensis.* Scale bars 2 µm. Images courtesy of Dr. Vit Hubka



7.5 Future directions

- Cats are an excellent model of a spontaneously occurring invasive form of aspergillosis in an immunocompetent host, and a translational approach could be taken to study similar aspergillosis syndromes in immunocompetent humans, including chronic granulomatous rhinosinusitis. In order to apply this translational approach it is important to understand the unique pharmacologic disposition of cats in the metabolism of antifungal drugs. Drugs that require hepatic glucuronidation, in which drugs are conjugated with glucuronic acid, catalysed by the uridine diphosphate glucuronosyltransferase (UGTs), are metabolized uniquely in cats compared to humans because of deficiency of certain UGT isoforms (Court, 2013). Posaconazole is one such drug, whose metabolism is mediated by UGT enzyme pathways. Its metabolism has been studied widely in a number of mammalian species, including humans, mice, rats, rabbits, dogs and cynomolgus monkeys, but not in cats (Nomeir et al., 2000). Although some treatment success has been documented in my studies on URTA using posaconazole, the pharmacokinetics and pharmacodynamics of this drug in cats, need to be determined. There is a need for the pharmacological disposition of many other antifungal drugs to be studied in cats, including the newer triazole drugs voriconazole, ravuconazole, pramiconazole, and isavuconazole, and the echinocandins, e.g. caspofungin. As a first step, my group has initiated a study to determine the pharmacological disposition of posaconazole in domestic cats.

- The predilection for pure-bred brachycephalic cats of Persian lineage, including Persians, Himalayans, Exotic shorthairs, British Shorthairs, Scottish shorthairs and Ragdoll cats is intriguing. Future studies are need to determine whether the predilection is a heritable defect in immunity or whether it is related to the abnormal bony conformation of the nasal cavity and paranasal sinuses in these breeds of cats. An approach similar to that taken by Professor Dominique Peeter's group at the University of Liege to study the immunopathogenesis of canine SNA could be taken. A first basic step would be to perform an

immunohistochemical study using archived formalin-fixed tissues to characterize the phenotype and distribution of leukocytes and immunoglobulins in the sinonasal mucosa and other affected tissues of cats with URTA (Peeters et al., 2005). Prospective studies would then be required to study both the innate and adaptive immune-response. Comparison of cytokine and chemokine mRNA expression, and of PRR mRNA expression from cats with URTA, from cats with other upper respiratory diseases, and from brachycephalic and non-brachycephalic healthy cats could be compared. Also, given the finding of SNPs in several PRRs in humans that increase susceptibility to aspergillosis, a mutational analysis of PRR genes could be performed in brachycephalic cats of Persian lineage, in cats with aspergillosis, and in healthy non-brachycephalic cats.

- *A. viridinutans* sensu stricto was first isolated from rabbit dung in Australia (McLennan et al., 1954) . All other members of the *A. viridinutans* complex have been isolated from Australia. *A. felis* is the only member of the *A. viridinutans* complex to be isolated from host animals but not from the environment. The environmental distribution of *A. felis* and other members of the *A. viridinutans* complex has not been studied in Australia. Therefore the risk of exposure in susceptible hosts is unknown. An environmental isolation study could be performed, starting with soil sample collection from the homes of cats diagnosed with aspergillosis in Australia.

- Given the discovery of a high proportion of *Aspergillus* section *Fumigati* isolates being cryptic species, and the innate antifungal resistance properties of these isolates, prospective molecular identification studies of fungal isolates from human patients with aspergillosis is warranted in Australia.

- I have investigated the computed tomographic features of aspergillosis in cats. Interestingly, the CT features of cryptococcosis, a more common mycosis than that of aspergillosis, have not been systematically reported. It is my clinical impression that sino-orbital cryptococcosis is quite common, but given the

excellent sensitivity and specificity of antigen tests for diagnosis of cryptococcosis, CT is usually not performed in affected cats. Finally, whether post-contrast features will aid in differentiating SOA from neoplasia in cats requires characterisation of the latter.

The incidence of aspergillosis and other invasive mycoses in companion animals and humans predicted to increase with global warming. This thesis provides an important step in increasing our understanding of aspergillosis by the characterisation, diagnosis and treatment of one form of aspergillosis in the domestic cat.

Chapter 8. References

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Appendix 1

FELINE SINO-ORBITAL ASPERGILLOSIS: AN EMERGING CLINICAL SYNDROME

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INTRODUCTION

Until recently, little information has been available regarding aspergillosis affecting the upper respiratory tract (URT) of cats. URT aspergillosis in cats is a more aggressive disease than canine sinonasal aspergillosis (SNA). Whilst some cats present with SNA characterised by sneezing and nasal discharge, there is a propensity for extension of infection beyond the sinonasal cavity to involve adjacent structures including the orbit, palate, nasopharynx and cribriform plate. Thus, the first presenting signs of URT aspergillosis in cats may be referable to orbital invasion resulting in sino-orbital aspergillosis (SOA), which can be considered a progression from SNA. The fungal pathogens identified in many feline cases, *Neosartorya* spp, are related to but distinct from the major canine pathogen, *Aspergillus fumigatus*.

ETIOLOGY

Classification of fungal pathogens

Both *Aspergillus* spp and *Neosartorya* spp are implicated as fungal pathogens in feline URT aspergillosis.[1,2] These two closely related genera are classified within *Aspergillus* section *Fumigati.* Both are ubiquitous, filamentous, saprophytic, ascomycetes distributed primarily in soil and decaying vegetation. Confusion may arise over nomenclature because fungi can exist in different physical states corresponding to different stages of the life cycle; the anamorphic, asexual stage is typically mould-like and bears conidia (spores) whereas the teleomorphic, sexual stage, produces fruiting bodies (cleistothecia) containing ascospores. The total organism is referred to as the holomorph. Some *Aspergillus* species are strictly anamorphic, and therefore reproduce by asexual means only. Teleomorphic species within *Aspergillus* section *Fumigati* have been assigned to the genus *Neosartorya. Aspergillus* section *Fumigati* currently includes 9 strictly anamorphic *Aspergillus* spp and 24 *Neosartorya* spp.[3,4] *Aspergillus fumigatus*, previously thought to be strictly anamorphic, was recently shown to have a fully functional sexual reproductive cycle that leads to the production of cleistothecia and ascospores. Its teleomorph is *Neosartorya fumigata*.[4]

Identification of fungal pathogens in cats

Prior to 2007, complete reports of feline URT aspergillosis have been limited to case reports describing infections in 13 cats.[5-13] Affected cats presented with signs referable to sinonasal cavity infection (SNA) or sino-orbital infection (SOA). The fungal pathogen was often not identified either because fungal culture was negative or was not performed. In 5 cases where fungal culture was positive, isolates were proposed to be *Aspergillus* spp in 4 cases and *Penicillium* spp in another.[5,6,11,13] Identification to species level based on fungal culture morphology was performed in only 2 cases where *A fumigatus* and *A niger* were documented.[11,13]

In 2006, 3 cases of feline URT aspergillosis were diagnosed at the author's institution. This prompted a medical record search, which identified only 3 cases in the preceding 19 year period (January 1986 to December 2005). Following publication of a description of the syndrome of SOA and a call for cases, a further 15 cases were recruited for study between 2006 and 2008. Clinicopathological data from the resulting cohort of 21 cats from Eastern Australia provides new information on this disease in cats.[1,2] Fungal pathogens were readily isolated; culture on Sabouraud's dextrose agar was attempted in all 21 cases and was positive in 20. On the basis of fungal colony morphology, *A fumigatus* was the most common isolate identified in this cohort. However, it is not possibly to correctly identify some species belonging to *Aspergillus* section *Fumigati* using morphological and cultural characteristics alone. Thus, molecular diagnostics were performed on the fungal cultures and/or on formalin-fixed paraffin embedded (PE) or fresh tissues from affected cats to confirm the identity of the isolates. A panfungal PCR that amplifies the internal transcribed spacer 1 (ITS1) region of the ribosomal DNA gene cluster between the 18S and 5.8S rRNA genes was used for PE or fresh tissues. A second panfungal PCR that targets a larger region of the rDNA gene cluster including the ITS1, 5.8S gene and ITS2 regions was performed on fungal cultures. The ITS1 regions was targeted because it is multicopy (≥ 100 copies in the fungal genome), universal fungal primers are available and it contains highly variable regions for species identification.[14] PCR products were sequenced and compared with those in the GenBank database.

Results of these molecular analyses indicated that the majority of feline isolates that had been identified as *A fumigatus* based on culture morphology were actually *Neosartorya* spp.[1,2] In several cats, *A fumigatus* was identified using both fungal colony morphology and PCR of culture material or tissue. PCR of fungal culture material and/or tissue obtained from canine SNA cases was also carried out at the same time and identified *A fumigatus* in all cases.[1] For *Neosartorya* spp isolates, in most cases it was not possible to distinguish between several possible species of *Neosartorya* using the panfungal PCR because of limited sequence variation in the ITS1 region. In one case, *N pseudofischeri* was identified from PCR of both culture material and fresh tissue with 100 percent identity to sequences in the GenBank database.

Because of limitations for species identification using morphological criteria alone, a polyphasic taxonomic approach is recommended for definitive identification of species within *Aspergillus* section *Fumigati*. This involves a combination of macro- and micromorphology, extrolite (fungal metabolite) profiles and PCR determination of targeted genes including β-tubulin, calmodulin, ITS and actin.[3]

EPIDEMOLOGY

No age or sex predilection for feline URT aspergillosis is apparent. Disease has been reported in 15 females and 18 males; most were desexed.[1,2,5-13] The median and mean age at diagnosis was 6 years with a range from 1 and ½ to 13 years. An intriguing finding is the preponderance of brachycephalic cats affected; 16 of 34 cases were brachycephalic, including 8 Persians and 6 Himalayans. This contrasts starkly with canine SNA where dolicocephalic or mesocephalic breeds are typically affected. The basis for this potential brachycephalic breed association in cats is not clear. Impaired drainage of URT secretions due to brachycephalic conformation may be important. In humans, decreased sinus aeration and drainage of respiratory secretions secondary to infection, polyps and allergic rhinosinusitis have been identified as risk factors for invasive SNA aspergillosis. It is likely that additional risk factors are present in brachycephalic cats, since brachycephalic dogs are under-represented for SNA. These may include heritable defects in mucosal immunity or common environmental factors such as antecedent viral URT infection.[11] Chronic FHV infection markedly alters sinonasal cavity architecture because of turbinate lysis secondary to intense inflammation, resulting in altered local mucosal defense mechanisms. One third of the Australian cohort of cats had a history of chronic recurrent rhinosinusitis before aspergillosis was diagnosed.[1,2] So far there is no evidence of a retroviral association with feline URT aspergillosis; of 19 affected cats tested for feline leukaemia virus antigen, only 1 was positive and all 19 cats tested for feline immunodeficiency virus were seronegative. Therefore, it appears that URT aspergillosis in cats occurs in apparently systemically immunocompetent individuals, some of which have identifiable breaches in local defence mechanisms. The apparent increased prevalence at our institution is likely a consequence of increased recognition of cases. Cases have been reported mostly in Eastern Australia and the USA with individual cases from Switzerland and the United Kingdom.[1,2,5-13]

PATHOGENESIS

Neosartorya spp have only rarely been associated with disease; isolated cases of invasive pulmonary infection, osteomyelitis, endocarditis, peritonitis and mycotic keratitis have been described in immunocompromised humans.[15] Mistaken identification of *Neosartorya* spp as *A fumigatus* is considered likely to have resulted in underestimation of disease caused by *Neosartorya* spp in humans.[15] Compared to SNA in dogs where fungal infection is usually confined to the sinonasal cavity, infections in cats have a tendency to become locally invasive with extension to involve the orbit.[1,2,5,6,8-10,13] In canine SNA fungal hyphae do not invade the nasal mucosa and are typically present within overlying superficial necrotic plaques. Like SNA in dogs, infection in cats starts in the sinonasal cavity. It is likely that SNA and SOA in cats represent a spectrum of disease, with SOA being a manifestation of more chronic, invasive infection. Progression from SNA to SOA has been documented in individual cases.[10] Furthermore, all cases of feline SOA to date have shown evidence of sinonasal cavity involvement on diagnostic imaging or at necropsy. [1,2,5,6,8-10,13] The orbital lamina, situated between the orbit and frontal sinus, is the most common anatomic region where extension of infection from the sinonasal cavity to the orbit has been identified.[2]

However, only some cases of SNA progress to SOA. The factors that determine whether infections will progress from SNA to SOA in individual cats have not yet been identified.

CLINICAL SIGNS

In dogs the triad of muzzle pain, profuse mucoid to haemorrhagic chronic nasal discharge and depigmentation, crusting or ulceration of one or both nares is highly suggestive of SNA. In cats, presenting signs of SNA are less specific. Most cats will have a history of sneezing and a unilateral or bilateral serous to mucopurulent nasal discharge.[1,2,7,11,12] Intermittent epistaxis occurs in 40%. In contrast to dogs, neither nasal depigmentation nor ulceration has been documented in cats with SNA. A discharging sinus or soft-tissue mass may be identified overlying the frontal sinus or the nasal bone as a result of bony lysis and fungal proliferation. Stertor is variably present due to excessive nasal secretions and/or a mass lesion in the caudal nasal cavity and/or nasopharynx.

Combining data from previously reported cases and the Australian cohort, 21 of 39 cats had SOA.[1,2,5-13,16] Although most cats with SOA have a history of sneezing or nasal discharge in the 6 months prior to presentation, at the time of presentation, nasal signs may be subtle or absent. Rather, cats with SOA typically present with a constellation of clinical signs referable to invasive expansion of a fungal granuloma in the ventromedial orbit. These include unilateral exophthalmos with third eyelid prolapse, exposure keratitis and conjunctival hyperemia and a mass or ulcer in the ipsilateral pterygopalatine fossa behind the last molar tooth.[1,2,13] Invasion through the palatine bone causes ulceration of the hard palate. Stertor is also common. In severe, chronic infections, exophthalmos can be bilateral.[1,2,5,6] Pain on opening the mouth is not usually present. Extension of infection outside the sinonasal cavity can also result in facial distortion, including swelling of the nasal bridge, periorbital tissues and soft tissues adjacent the maxilla. Neurological signs are uncommon but develop subsequent to CNS invasion through the cribriform plate or sphenoid sinus. Submandibular lymph node enlargement and pyrexia are also common. In contrast, cats with SNA are not usually pyrexic.

DIAGNOSIS

Definitive diagnosis of URT aspergillosis in cats is achieved by cytological or histological identification of fungal hyphae in affected tissue together with identification of the fungal pathogen. Obtaining a definitive diagnosis may require various combinations of diagnostic tests including radiography, CT, endoscopy, cytology, serology, fungal culture and molecular techniques. Serum anti-*Aspergillus* antibodies can be detected by counter-immunoelectrophoresis (CIE), agar gel immunodiffusion (AGID) or ELISA. The diagnostic utility of serum anti-*Aspergillus* antibody detection in cats with URT aspergillosis has not been evaluated. Two of 3 cats with SNA tested positive for serum antibodies against *A fumigatus* using CIE and another cat with SNA tested positive using

AGID.[7,11] Galactomannan (GM), a water-soluble polysaccharide cell wall component of *Aspergillus* and some other fungal species, is released in variable quantities during hyphal growth in tissues. This antigen can be detected in the serum of human patients with invasive *Aspergillus* infections using a commercially available sandwich ELISA (Platelia™ *Aspergillus*). GM antigen levels correlate with the tissue fungal burden and can be used to monitor clinical outcomes and response to therapy.[17] Rising GM levels are associated with treatment failures in humans. The use of GM assays for diagnosis and monitoring of feline URT aspergillosis is currently under investigation in our laboratory.

For investigation of suspected sinonasal cavity or sino-orbital disease, CT is the imaging modality of choice. Reported changes in mycotic rhinosinusitis in cats include lysis of turbinates, palatine bone, nasal septum, frontal bone and/or cribriform plate.[16] Nasal cavity involvement is usually bilateral. CT findings on cats with SOA within the Australian cohort included punctate lysis of the orbital lamina, ventromedial soft tissue orbital mass, opacification of the sphenoid and frontal sinuses due to fluid or soft-tissue, and a soft-tissue mass in the choanae or nasopharynx.[2] Sinonasal cavity involvement in SOA is usually bilateral. In cats with URT aspergillosis differentiation of invasive mycotic disease from malignant neoplasia may not be possible from imaging findings alone.[16]

Appendix 1

TREATMENT AND PROGNOSIS

The typical antifungal susceptibility profile of *Neosartorya* spp isolated from cats with aspergillosis includes susceptibility to amphotericin B (AMB) and posaconazole (PCZ), intermediate or dose-dependent susceptibility to itraconazole (ICZ) and voriconazole (VCZ), and resistance to 5-flucytosine, ketoconazole and fluconazole. Elevated minimum inhibitory concentrations of VCZ to *Neosartorya* spp have been documented elsewhere.[15] In contrast, both VCZ and PCZ typically have greater activity than ICZ *in vitro* against *A fumigatus*.[18] However, the correlation between *in vitro* antifungal susceptibility test results and *in vivo* response to therapy is only modest at best. For treatment of invasive aspergillosis in humans, VCZ has replaced AMB as the drug of choice for first-line therapy. PCZ is used for antifungal prophylaxis in high-risk patients and for salvage therapy. The echinocandins, including caspofungin, are reserved for salvage therapy or combination antifungal therapy for refractory invasive aspergillosis.[19] Combination antifungal therapy may provide enhanced efficacy compared to single drug therapy for treatment of invasive aspergillosis.[20,21]

URT aspergillosis in cats is more difficult to treat than SNA in dogs. Treatment of SNA carries a better prognosis than for SOA.[2] The prognosis for resolution of SOA is generally poor. Antifungal triazole drugs are the mainstay of therapy for SNA, given alone or in combination with terbinafine or AMB. [1,2,11,12] AMB can be administered as amphotericin deoxycholate (AMB-D) or as liposomal AMB (AMB-L).[2] ICZ or PCZ are the triazoles of choice, although the pharmacokinetics and pharmacodynamics of the latter are yet to be studied in cats. Single topical sinonasal clotrimazole (CLT) infusions have been reported in 3 cats.[10,11,13] In one cat where infection was confined to the sinonasal cavity treatment with oral ICZ and a 1 per cent CLT infusion was successful.[11] In 2 other cats with SOA, a CLT infusion was part of a multimodal therapeutic approach. One cat was euthanased because of suspected progression of infection despite treatment with ICZ and radical surgical debridement of the orbit, including extenteration of the globe.[10] In another cat treatment with ICZ, topical CLT and AMB-D did not resolve infection. However, the cat was cured after treatment with oral PCZ administered for 16 weeks.[13] The use of topical CLT infusions should be restricted to treatment of cats with SNA where there is an intact cribriform plate, no evidence of orbital involvement on CT, and no histological evidence of submucosal fungal invasion.[2] Concomitant systemic antifungal therapy with ICZ or PCZ is also recommended.

The optimal treatment regimen for SOA in cats has not been identified. Of 14 cases where treatment was attempted, infection resolved in only 4.[2,13] Three of those were treated with medical therapy alone, including 1 which resolved after PCZ administration for 16 weeks.[13] In another cat, infection resolved after 7 months of combination therapy using ICZ and AMB-D (cumulative dose 11 mg/kg) and terbinafine. A third cat responded well to 8 months of therapy with ICZ and AMB-D (cumulative dose 14 mg/kg). However, clinical and CT evidence of infection recurred 3 weeks after ICZ was stopped. Treatment was reinstituted with terbinafine and PCZ for 4 months, followed by PCZ alone for a further 3 months. Signs resolved and the cat remained asymptomatic 1 year later. The fourth case was treated with surgical debridement of the frontal sinuses during diagnostic investigation followed by AMB-D (cumulative dose 10 mg/kg) and ICZ for 7 months. Clinical signs of infection resolved and the cat was asymptomatic 2 years later. Treatment failures occurred in 4 cats which were treated with medical therapy alone and in 6 cases treated with medical therapy and radical surgical debridement of orbital granulomas, including exenteration in 5 cases.[2] Ongoing studies of the pathogenesis of URT aspergillosis in cats, combined with pharmacokinetic/pharmacodynamic data for newer antifungals in this species will assist in identifying the optimal therapeutic approach to treatment of this disease.

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KEYWORDS
Neosartorya, Aspergillus fumigatus, respiratory mycoses, fungal infections

FELINE UPPER RESPIRATORY ASPERGILLOSIS: HOW DIFFERENT IS IT FROM CANINE SINONASAL ASPERGILLOSIS?

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Introduction

Canine sinonasal aspergillosis (SNA) was first reported in 1897 while reports of a similar disease in cats did not appear until the 1980s (1-2). Upper respiratory tract aspergillosis (URTA) is an emerging feline infection with over two-thirds of the approximately 50 reported cases being published in the last 5 years. In dogs the major form of disease (>99% of cases) is SNA, while in cats sino-orbital aspergillosis (SOA), is the more common form (63% of cases).

Aetiology - Controversies in fungal nomenclature and identification (ID)

More than 250 species have been ascribed to the Genus *Aspergillus*. Isolates from canine and feline URTA are usually from the subgenus *Fumigati* section *Fumigati* (the *Aspergillus fumigatus* complex) (3-4).This complex contains asexual members (anamorphs), many of which also have sexual forms (teleomorphs). Teleomorphs have been assigned to a different genus – *Neosartorya*. Classical nomenclature requires that an organism with alternate names be labeled with that of its sexual phase. This created confusion for organisms such as *A. fumigatus* where the teleomorph (*Neosartorya fumigata*) was only recently discovered (5). In sweeping reforms to the nomenclature code a “one-fungus, one-name” principle was adopted in 2011 (6). In the revised code (to be published in 2013) it is likely that the oldest generic name (eg. *A. fumigatus*), irrespective of whether it is typified by a species name with a teleomorphic or an anamorphic type, will be used. Members of the *A. fumigatus* complex cannot be reliably identified using phenotypic features alone. *A. fumigatus*-mimetic species have very similar anamorph colony morphology to *A. fumigatus.* Of 86 isolates from human patients with invasive aspergillosis previously identified as *A. fumigatus*, 12 were subsequently identified on comparative partial sequence analyses of genes encoding beta-tubulin and rodlet A as *A. udagawae* (*Neosartorya udagawae*) (7). In this subset of patients the median duration of illness was 7 times longer and disease was refractory to standard therapy. *A. fumigatus*-mimetic species have higher *in vitro* minimum inhibitory concentrations for amphotericin-B (AMB) and triazole antifungal drugs than *A. fumigatus*.

Aetiology - Advances in ID of fungal pathogens in feline and canine URTA

Isolates from 23 Australian cats with URTA were identified using PCR and sequencing of the Internal Transcribed Spacer (ITS) region (3). *A. fumigatus* was identified in 4 of 6 cats with SNA while all 17 isolates from cats with SOA were identified as *A. fumigatus*-mimetic species. Individual species determination was not possible using ITS sequence analysis alone. In a subsequent study of these and other isolates at the CBS KNAW Fungal Biodiversity Centre in Utrecht, using sequence comparisons of the beta-tubulin, calmodulin and rodlet A regions, a single novel species of *Aspergillus* was identified from the majority of cats with SOA and from 1 cat with SNA (unpublished). *A. udagawae* was identified as a cause of feline SOA in a single case report from Japan (8). Current evidence suggests that *A. fumigatus* is the most common cause of feline SNA and that SOA is only caused by *A. fumigatus*-mimetic species. Phenotypic ID of a large number of isolates from dogs with SNA has implicated *A. fumigatus* as the most common aetiologic agent (4). Molecular ID has been performed on a small number of isolates only (3,9). In 14 dogs with SNA, DNA of *Penicillium* or *Aspergillus* spp. was detected in nasal mucosal biopsies using a genus-specific real-time PCR assay (9). Species specific PCRs to

detect DNA of *A. fumigatus, A.niger, A. terreus* or *A. flavus* were positive for *A. fumigatus* in 7 dogs and negative in the other 7 dogs. These isolates could have been *Penicillium* spp. or another *Aspergillus* spp. or there may have been insufficient *A. fumigatus* DNA present to be positive with the specific PCR. In another study, ITS sequencing of archival tissues from 7 dogs with SNA identified *A. fumigatus* in all 7 cases (3).

Pathogenesis

URTA in cats and dogs starts in the sinonasal cavity. Extension of infection into adjacent structures including the orbit and paranasal tissues occurs in SOA, as evidenced by progression of SNA to SOA (10), sinonasal cavity involvement on imaging or at necropsy in cases of SOA (3) and by isolation of the same novel species of *Aspergillus* that causes SOA in a case of SNA . Infecting fungal species may be a major determinant of progression. Canine SNA is a non invasive mycosis - fungal hyphae do not invade the nasal mucosa and are located in adjacent superficial necrotic plaques (9). In feline SOA fungal hyphae invade the respiratory epithelium and form granulomas within the orbit and paranasal tissues.

Epidemiology

In both cats and dogs URTA typically occurs in young to middle-aged animals. Of 49 reported feline cases the mean age at diagnosis was 6.5 years (range 1.5 -13 years). In case-series of canine SNA, mean age at diagnosis was 5 to 6 years. No sex-predisposition has been identified for feline URTA whereas males are over-represented in canine SNA. In feline URTA brachycephalic breeds (mostly Persian or Himalayan) account for 40% of cases. By contrast dolicocephalic and mesaticephalic breeds of dogs are predisposed to SNA. Overall (61%) cats with SNA were brachycephalic while (33%) cases of SOA were brachycephalic. Most cats and dogs with URTA are systemically healthy. Risk factors for URTA in humans include decreased sinus aeration and drainage secondary to infection, polyps and allergic rhinosinusitis. Reduced drainage of URT secretions due to brachycephalic conformation could be a risk factor in cats. However, since brachycephalic dogs are under-represented for SNA, it is likely that additional risk factors are present in cats, eg. heritable defects in mucosal immunity, previous viral URT infection and previous antibiotic treatment favouring fungal colonisation (11).

Clinical Presentation

In dogs the triad of muzzle pain, profuse mucopurulent to haemorrhagic nasal discharge and depigmentation, crusting or ulceration of one or both nares is highly suggestive of SNA. Clinical signs (CS) in feline SNA are more subtle and include sneezing, mucopurulent nasal discharge and intermittent epistaxis (40% of cases). Occasionally there is a cutaneous sinonasal discharging sinus or soft-tissue mass. Cats with SOA are presented for unilateral exophthalmos and other CS referable to invasive expansion of a retrobulbar fungal granuloma: dorsolateral deviation of the globe, conjunctival hyperaemia, third eyelid prolapse, exposure keratitis and an oral mass or ulcer in the ipsilateral pterygopalatine fossa behind the last molar tooth. Extension of infection into paranasal tissues may cause facial distortion. Nasal signs are absent in 40% of cases but the medical history usually reveals sneezing or nasal discharge in the previous 6 months. Neurological signs (15% of cases) include blindness, circling, facial muscle fasciculation and hyperesthesia.

Diagnosis

Diagnosis of feline and canine URTA may require various combinations of serology, computed tomography, endoscopy, cytology, histology and fungal culture. The sensitivity and specificity of

anti-*Aspergillus* antibody detection for the diagnosis of feline URTA is not known. Five of 10 reported cases (9 SNA, 1 SOA) tested seropositive (2-3, 11-13). By contrast, two recent studies evaluated serology for diagnosis of canine SNA (4, 14). In one study agar gel immunodiffusion (AGID) was compared with fungal culture of nasal biopsies (4) and in the other antibody detection by AGID and ELISA were compared (14). Both methods used a purified aspergillin preparation composed of *A. fumigatus*, A. *niger* and *A. flavus* extracts (14). Fungal culture was more sensitive (81%) than serology (67%) while specificity was high for both fungal culture (100%) and serology (98%) (4). In the second study ELISA had higher sensitivity (88%) than AGID (76%) and specificity was high for both methods (ELISA 97%, AGID 100%) (14). Seropositivity for *Aspergillus* spp. is highly suggestive of SNA in dogs but negative test results do not rule out aspergillosis. Serum galactomannan (GM) measurement for diagnosis of feline URTA was recently evaluated using the Platelia™ ELISA (15). GM was measured in cats with URTA (n=13; 6 SNA, 7 SOA), other URT disease (n=15), β-lactam antibiotic treated cats (n=14) and healthy cats (n=44). Overall sensitivity was 24% and specificity was 78% for a cut-off optical density index (ODI) of 1.5. High numbers of false positive results were identified in young cats and antibiotic-treated cats. Using the same ELISA, GM was measured in dogs with SNA (14). Using a cut-off ODI of 0.5, 24% of dogs with SNA tested positive, as did 11% of dogs with nasal tumours, 9% of dogs with non-fungal rhinitis and 24% of healthy dogs. Overall sensitivity was 24% and specificity was 82%. Currently available antigen tests cannot be recommended in the evaluation of feline or canine patients with URTA.

Treatment

Overall the prognosis for treatment of feline and canine SNA is good. Topical therapy is preferred for canine SNA because of direct contact with fungal plaques, although multiple applications are often required. In a multicentre, retrospective study of 81 dogs treated with topical clotrimazole or enilconazole using catheters placed intranasally and/or via sinus trephination, a single treatment was successful in 47% (16). Techniques reported to improve efficacy of topical antifungals include endoscopic/sinuscopic debridement of fungal plaques, endoscopic guidance of infusion catheters into the caudal frontal sinus and use of depot preparations (eg 1% clotrimazole or bifonazole cream). Of 14 feline SNA cases with available follow-up, signs resolved in 11 (78%) (2-3, 11-13). The most common successful treatment regimes were systemic antifungal therapy alone (triazole +/- AMB) (n=5), systemic triazole therapy and topical intranasal clotrimazole (n=2) or topical therapy alone (single 1% intranasal clotrimazole infusions) (n=2). Few cases of feline SOA have been treated successfully despite aggressive therapy including orbital exenteration and systemic fungicidal antifungals (3, 17-18). Posaconazole or itraconazole are recommended for first-line therapy. Use of triazole antifungals in combination with AMB may improve outcomes. Voriconazole should not be used initially due to a high frequency of adverse neurological events (19). Molecular ID of pathogens causing feline URTA will facilitate development of optimal treatment protocols.

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Conflict of interest statement

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CHAPTER

5 Upper Respiratory Tract Aspergillosis

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CHAPTER OUTLINE

Although most small animal practitioners will be familiar with the clinical syndrome of canine sinonasal aspergillosis (SNA), little information has been available regarding aspergillosis affecting the upper respiratory tract (URT) of cats. Informative recent studies have highlighted major species differences that have implications for the recognition and treatment of this disease in this species. In cats, URT aspergillosis often is much more aggressive than its canine counterpart.

Although some cats present with SNA characterized by sneezing and nasal discharge, there is a propensity for the infection to extend beyond the sinonasal cavity to involve adjacent structures including the orbit, palate, nasopharynx, and cribriform plate. Therefore the first presenting signs of URT aspergillosis in cats may be referable to orbital invasion resulting in sino-orbital aspergillosis (SOA), which can be considered a progression from SNA.

Clinical signs in SOA are the result of an invasive retrobulbar fungal granuloma and include exophthalmos, prolapse of the nictitating membrane, exposure keratitis, a lesion (mass or ulcer) in the pterygopalatine fossa, and stertor.

The fungal pathogens identified in the majority of feline cases, *Neosartorya* spp., are related to, but distinct from, the major canine pathogen, *Aspergillus fumigatus*. Until more information is available on the most effective treatment for feline aspergillosis, the prognosis remains guarded, especially for SOA. Topical antifungal triazole therapy using clotrimazole infusions in combination with an oral antifungal triazole is recommended for early SNA. In all other cases, systemic antifungal therapy using combinations of triazoles, amphotericin B, and terbinafine is indicated.

ETIOLOGY

Until recently, reports of feline URT aspergillosis have been limited, describing infections in a total of 13 cats.[1-9] The affected cats presented with signs referable to sinonasal cavity infection (eight cases) or sino-orbital infection (five cases). The fungal pathogen often was not identified in these reports, either because fungal culture was negative or was not performed. In five cases in which fungal culture was positive, isolates were proposed to be *Aspergillus* spp. in four cases and *Penicillium* spp. in another.[1,2,6,8,9] Identification to species level based on morphological and cultural characteristics was performed in only two cases, in which *A. fumigatus* and *A. niger* were documented.[8,9]

In 2006 three cases of feline URT aspergillosis were diagnosed at the authors' institution. This prompted a search of our medical records, which identified only three cases in the preceding 19-year period (January 1986 to December 2005). Following publication of a description of the syndrome of SOA and a call for cases, we actively recruited 15 additional cases for prospective study between 2007 and 2008.[10] Clinicopathological data from the resulting cohort of 20 cats from Eastern Australia provide exciting new information on this disease in cats.[11] Fungal pathogens were cultured readily. Culture was attempted in all 21 cases and was positive in 19.

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Sabouraud's dextrose agar was used with addition of gentamicin and chloramphenicol when bacterial contamination of diagnostic samples was suspected. Cultures were incubated at 37° C and 28° C. On the basis of colony morphology, *A. fumigatus* was the most common isolate identified in this cohort. However, it is important to understand that the correct identification of some species of fungi belonging to *Aspergillus* section *Fumigati* can not be achieved using morphological and cultural characteristics alone.[12,13] Therefore molecular diagnostics were performed on the fungal cultures and/or on formalin-fixed paraffin embedded (PE) or fresh tissues from affected cats to confirm the identity of the isolates. A panfungal polymerase chain reaction (PCR) that amplifies the internal transcribed spacer 1 (ITS1) region of the ribosomal DNA gene cluster between the 18S and 5.8S rRNA genes was used for PE or fresh tissues. A second panfungal PCR that targets a larger region of the rDNA gene cluster including the ITS1, 5.8S gene, and ITS2 regions was carried out on material obtained from fungal culture. These regions of the fungal genome were chosen because they are multicopy (= 100 copies in the fungal genome) and because they contain highly variable regions facilitating species identification in some cases. Polymerase chain reaction products were sequenced and compared with those in the GenBank database.[14,15]

Significantly, results of these molecular analyses indicated that the majority of feline isolates that had been identified as *A. fumigatus* based on culture morphology were actually *Neosartorya* spp. (Table 5-1). In most cases, it was not possible to classify the fungal pathogen further using the panfungal PCR because of limited sequence variation in the ITS1 region; for example, it was not possible to distinguish between *N. udagawae, N. fischeri,* and *N. aureola*. In one case, *N. pseudofischeri* was identified from PCR of both culture material and fresh tissue with 100 per cent identity to sequences in the GenBank database. In four cats, *A. fumigatus* was identified using both fungal colony morphology and PCR of culture material or tissue. In three of these cats, disease was confined to the sinonasal cavity. Polymerase chain reaction of fungal culture material and/or tissue obtained from seven canine SNA cases also was carried out and identified *A. fumigatus* in all cases.[14] These findings suggest that *A. fumigatus* possibly may be associated with less invasive disease. Our experience has demonstrated that, unlike SNA in dogs, the most common isolates in URT aspergillosis in cats are *Neosartorya* spp. and that molecular diagnostic tests are required for the correct identification of these fungal pathogens.

CLASSIFICATION OF FUNGAL PATHOGENS

Both *Aspergillus* spp. and *Neosartorya* spp. are implicated as fungal pathogens in feline URT aspergillosis. These two genera are very closely related, and a brief review of their taxonomy is pertinent. Both are classified within *Aspergillus* section *Fumigati*. These ubiquitous, filamentous, saprophytic ascomycetes are distributed primarily in soil and decaying vegetation.[16] Confusion may arise over nomenclature because fungi can exist in different physical states corresponding to different stages of the life cycle; the anamorphic, asexual stage typically is moldlike and bears conidia (spores), whereas the teleomorphic, sexual stage is characterized by the production of fruiting bodies (cleistotheica) containing ascospores. The total organism is referred to as the *holomorph*. Some Aspergillus species are strictly anamorphic and therefore reproduce by asexual means only. Teleomorphic species within *Aspergillus* section *Fumigati* have been assigned to the genus *Neosartorya*. *Aspergillus* section *Fumigati* currently includes 9 strictly anamorphic *Aspergillus* spp. and 24 *Neosartorya* spp.[16,17] *Aspergillus fumigatus,* previously thought to be strictly anamorphic, was shown recently to have a fully functional sexual reproductive cycle that leads to the production of cleistothecia and ascospores. Its teleomorph is *Neosartorya fumigata*.[17a]

Species implicated in feline URT aspergillosis are listed in Table 5-2. *Neosartorya* spp. can exist in both anamorphic and teleomorphic states. The anamorphic states of some *Neosartorya* spp. can be mistaken for *A. fumigatus,* if identification is based on phenotypic characteristics such as thermotolerance and conidiophore morphology.[13,14] Phenotypic identification of *Neosartorya* spp. can be achieved partially from its teleomorph, including mor-

Table 5-1 Comparison of Fungal Culture Morphology versus PCR of Fungal Culture and/or Formalin-Fixed Paraffin-Embedded Tissues (PE) or Fresh Tissue for Fungus Identification (ID) in 18 Cases

Number of Cases	SOA or SNA	Fungal Culture Morphology ID	PCR of Fungal Culture ID	PCR of PE or Fresh Tissue ID
1	SNA	*A. fumigatus*	ND	*A. fumigatus*
3	SNA (2) SOA (1)	*A. fumigatus*	*A. fumigatus*	ND
1	SNA	*A. fumigatus*	*N. fischeri/ N. aureola / N. udagawae*	ND
1	SOA	Negative	ND	*A. lentulus/Neosartorya* spp.
1	SOA	*A. fumigatus*	*N. pseudofischeri*	*N. pseudofischeri*
12	SOA	*A. fumigatus*	*N. fischeri/ N. aureola / N. udagawae*	ND (n = 7) Negative (n = 2) A. *lentulus/N. fischeri* (n = 2)

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phology of ascospores. However, induction of the teleomorphic state of *Neosartorya* spp. to produce these fruiting bodies can be achieved only under specific growth conditions with specialized media. Even under optimal conditions, some species may not produce fruiting bodies in the laboratory.[14] Because of these limitations for species identification using morphological criteria alone, a polyphasic taxonomic approach is recommended for identification of species within *Aspergillus* section *Fumigati*. This involves a combination of macromorphology and micromorphology, extrolite (fungal metabolite) profiles, and PCR determination of gene sequences including ß-tubulin, calmodulin, ITS, and actin.[16,18] The phylogenetic relatedness of species belonging to *Aspergillus* section *Fumigati*, including pathogens implicated in feline URT aspergillosis based on ß-tubulin sequence data, is depicted in Figure 5-1.

Table 5-2 Species Belonging to *Aspergillus* Section *Fumigati* that Have Been Implicated in Feline Sinonasal and Sinorbital Aspergillosis[16]

Strict Anamorphic Species	**Teleomorph**
Aspergillus lentulus	None
Aspergillus viridinutans	
Anamorph	**Teleomorphic Species**
Aspergillus aureoluteus	*Neosartorya aureola*
Aspergillus fischeranus	*Neosartorya fischeri*
Aspergillus thermomutatus	*Neosartorya pseudofischeri*
Aspergillus udagawae	*Neosartorya udagawae*
Aspergillus fumigatus	*Neosartorya fumigatu*

EPIDEMIOLOGY AND RISK FACTORS

No age or sex predilection for feline URT aspergillosis is apparent. Disease has been reported in 15 females and 18 males; most were neutered. The median and mean age at diagnosis was 6 years, with a range from 18 months to 13 years.[1-9,11] An intriguing finding is the preponderance of brachycephalic cats affected; sixteen of 34 cases were brachycephalic, including eight Persians and six Himalayans. This contrasts starkly with canine SNA, in which dolichocephalic or mesocephalic breeds are typically affected.[19] The basis for this potential brachycephalic breed association in cats is not clear. Impaired drainage of URT secretions due to brachycephalic conformation may be important. Certainly, decreased sinus aeration and drainage of respiratory secretions secondary to infection, polyps, and allergic rhinosinusitis have been identified as risk factors for invasive SNA aspergillosis in human beings.[20] It is likely that additional risk factors are present in brachycephalic cats because brachycephalic dogs are

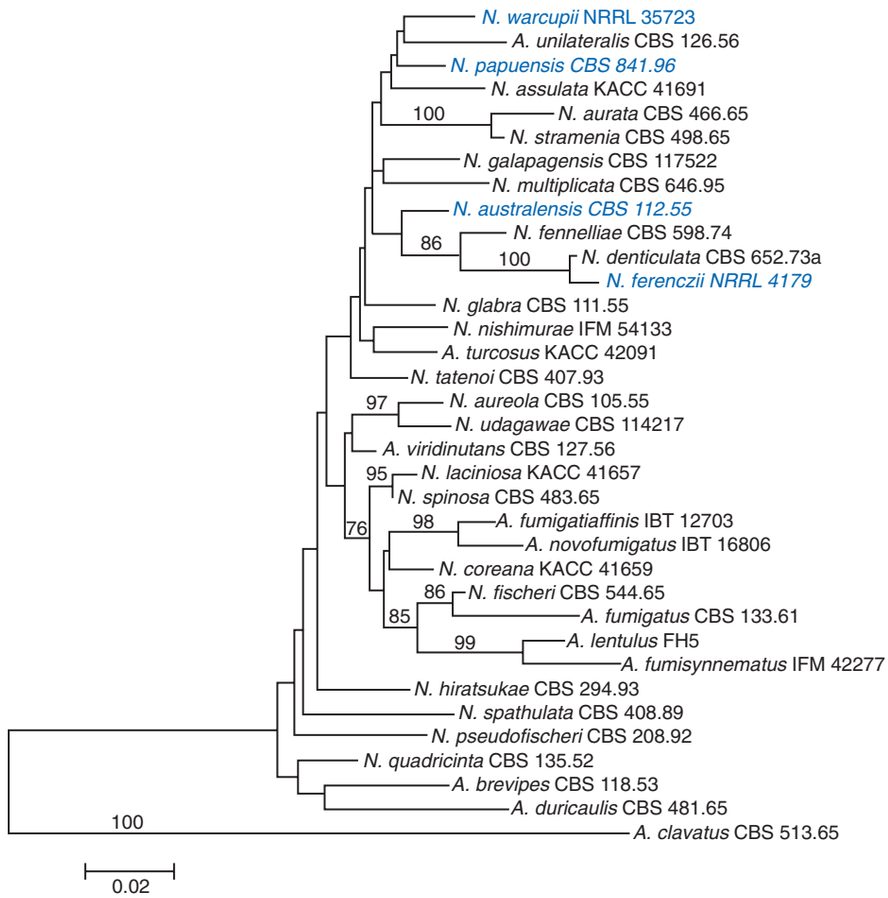

Figure 5-1 Based on ß-tubulin sequence data, the phylogenetic relatedness of species belonging to *Aspergillus* section *Fumigati,* including pathogens implicated in feline upper respiratory aspergillosis is depicted.

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underrepresented for SNA. These factors may include heritable defects in mucosal immunity or common environmental factors such as antecedent URT infection. Previous viral URT infection by feline calicivirus (FCV) or feline herpesvirus-1 (FHV) has been suggested as a possible risk factor for aspergillosis in cats.[7] In particular, chronic FHV infection can alter sinonasal cavity architecture severely because of turbinate lysis secondary to intense inflammation, resulting in altered local mucosal defense mechanisms. Six of 20 cats in one study had a history of chronic recurrent rhinosinusitis before aspergillosis was diagnosed, and FHV infection was confirmed by PCR in one cat.[11] Other possible risk factors identified were craniofacial trauma, diabetes mellitus, and a grass seed foreign body. There is no evidence currently of a retroviral association with feline URT aspergillosis; of 19 cats with aspergillosis tested for feline leukemia virus (FeLV) antigen, only one was positive, and all 19 cats tested for feline immunodeficiency virus (FIV) were seronegative.[3,5-9,11] Therefore it appears that URT aspergillosis in cats occurs in apparently systemically immunocompetent individuals, some of whom have identifiable breaches in local defense mechanisms.

Information on the prevalence and geographic distribution of feline URT aspergillosis is incomplete but is likely to be forthcoming now that the syndrome of SOA has been described.[10] The apparent increased prevalence at our institution is likely a consequence of increased recognition of cases. Cases have been reported most commonly in Eastern Australia (including 12 cats domiciled in New South Wales, two in Victoria, and seven in Queensland) and the United States, with individual cases from Switzerland and the United Kingdom.[1-3,5-9,11]

PATHOGENESIS

Aspergillus spp. and *Neosartorya* spp. are ubiquitous and their spores are inhaled readily. *Aspergillus* spp. can cause localized sinusitis in immunocompetent human beings or severe systemic disease as an opportunistic pathogen in immunocompromised human patients. *Neosartorya* spp. have been associated only rarely with disease; isolated cases of invasive pulmonary infection, osteomyelitis, endocarditis, peritonitis, and mycotic keratitis have been described in immunocompromised human beings.[13] The difficulty in distinguishing *A. fumigatus* from *Neosartorya* spp. using morphological criteria is likely to have resulted in underestimation of the frequency of disease caused by *Neosartorya* spp.[13]

Compared to SNA in dogs in whom fungal infection generally is confined to the sinonasal cavity, infection in cats appears to have a greater tendency to local invasion, with extension to involve the orbit. Of the 34 reported cases, 14 cats had SNA and 20 had SOA.[1,2,5,6,9,11] A brief consideration of mycotic rhinosinusitis syndromes in human beings has comparative significance because these syndromes can be either invasive or noninvasive. Invasive mycoses include acute (necrotizing) invasive fungal sinusitis, chronic invasive fungal sinusitis, and chronic granulomatous fungal rhinosinusitis. Noninvasive infections include fungal ball or sinus aspergilloma, allergic

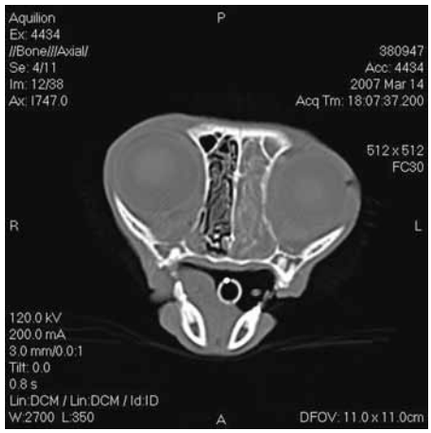

Figure 5-2 Transverse CT of skull of a cat with SOA. There is a right retrobulbar mass and diffuse opacification of the left nasal cavity. Punctate areas of lysis are visible in the right orbital lamina.

fungal sinusitis, and chronic erosive noninvasive fungal sinusitis.[21-24] Canine SNA bears many similarities to chronic erosive noninvasive fungal sinusitis in human patients and recently has been proposed as a model for studying the immunopathogenesis of disease in people.[24] *A. fumigatus* is the most common pathogen in dogs, although *A. niger, A. nidulans,* and *A. flavus* are implicated occasionally. *A. fumigatus* or *A. flavus* are isolated most commonly from human patients.[19,22,,24] Neither dogs nor human beings with this type of SNA are immunocompromised systemically. Fungal hyphae do not invade the nasal mucosa and typically are present within overlying superficial necrotic plaques. There is a mixed mononuclear inflammatory response within the often ulcerated nasal mucosa that contains lymphocytes and plasma cells predominantly, with some macrophages.[19,21,22]

Like SNA in dogs, infection in cats starts almost certainly in the sinonasal cavity. It is likely that SNA and SOA in cats represent a spectrum of disease, with SOA being a manifestation of more chronic, invasive infection and progression from SNA. Progression from SNA to SOA has been documented in individual cases.[6] However, not all cases of SNA progress to SOA. Virulence factors of the fungal pathogen, including toxic secondary metabolites, are likely to be involved. Gliotoxin is considered an important virulence factor in invasive aspergillosis in human patients. All 20 cases of SOA in cats showed evidence of sinonasal cavity involvement on diagnostic imaging or at necropsy.* The orbital lamina, situated between the orbit and frontal sinus, is the most likely region where extension of infection from the sinonasal cavity to the orbit occurs. Computed tomographic (CT) examination of the skull in cases of SOA frequently identifies punctate areas of lysis in the orbital lamina (Figure 5-2). In one case of SOA, a fistula was identified at surgery in the dorsomedial aspect of the orbit, communicating with the ipsilateral frontal sinus.[11] Systemic dissemination of disease is rare. A single case of feline SOA with

*References 1, 2, 5, 6, 9, 11.

concurrent pulmonary involvement has been reported. The retroviral status of that cat was unknown.[1]

SOA in cats bears a striking clinical and histological resemblance to chronic granulomatous sinusitis in people. The latter is an invasive mycosis caused by *A. flavus* or *A. fumigatus* that occurs in immunocompetent human beings subjected to hot, dry environmental conditions and poor hygiene. Most cases have been reported in Asia and Africa, predominantly in agricultural workers. The nasal cavity or sinuses invariably have been implicated as the primary site of infection following inhalation of fungal spores. A fungal granuloma forms within the sinuses. These granulomas are relatively avascular, consisting of a highly cellular inflammatory infiltrate of epithelioid macrophages ("giant cells"), histiocytes, plasma cells, and fungal hyphae. They may be necrotic or fibrotic and tend to invade contiguous structures such as the orbit or brain. Unilateral exophthalmos is common at presentation.[20,23] The histological features of URT aspergillosis in cats are not fully characterized. Both lymphoplasmacytic and suppurative noninvasive rhinitis have been described for infections confined to the sinonasal cavity.[7,8] SOA is clearly invasive, and we have found evidence of submucosal infection in the sinonasal cavity of affected cats.[11] In feline SOA, the granulomas are characterized by a necrotic center containing fungal hyphae. This is surrounded by granulation tissue that may be fibrous, infiltrated with neutrophils, lymphocytes, plasma cells, and eosinophils.[1,2,9] Commonly, we have seen retrobulbar granulomas surrounding the optic nerve. They typically feature a necrotic center containing branching septate PAS-positive fungal filaments and an inflammatory infiltrate of sheeting epithelioid macrophages with variable numbers of lymphocytes, plasma cells, and neutrophils extending into a zone of peripheral fibrosis (Figure 5-3). These fungal granulomas are well vascularized and in some cases are accompanied by heavy infiltrates of eosinophils.

HISTORY AND CLINICAL SIGNS

SINONASAL ASPERGILLOSIS

In dogs, the triad of muzzle pain, profuse mucoid to hemorrhagic chronic nasal discharge and depigmentation, and crusting or ulceration of one or both nares is highly suggestive of SNA. In cats, presenting signs of SNA are less specific. Most affected cats will have a history of sneezing and a unilateral or bilateral serous to mucopurulent nasal discharge that usually can be detected on physical examination (Figure 5-4). Intermittent epistaxis occurs in 40 per cent of cats with SNA. Interestingly, and in contrast to dogs, neither nasal depigmentation nor ulceration has been documented to date in cats with SNA. A discharging sinus or soft tissue mass may be identified overlying the frontal sinus or the nasal bone as a result of bony lysis and fungal proliferation (Figure 5-5). Facial distortion may be a feature.

Owners should be questioned specifically regarding any URT noise because this may be subtle, and the pattern of respiration should be evaluated carefully on physical examination. If it can be established that the cat has a stertor, at least part of the disease process can be localized to the caudal nasal cavity and/or nasopharynx. The stertor in SNA may result from excessive nasal secretions and/or a mass lesion in the caudal nasal cavity and/or nasopharynx (Table 5-3).

SINO-ORBITAL ASPERGILLOSIS

Although most cats with SOA have a history of sneezing or nasal discharge in the 6 months prior to presentation, it is important to note that, at the time of presentation, nasal signs may be subtle or absent. Rather, cats with SOA present typically with a constellation of clinical signs

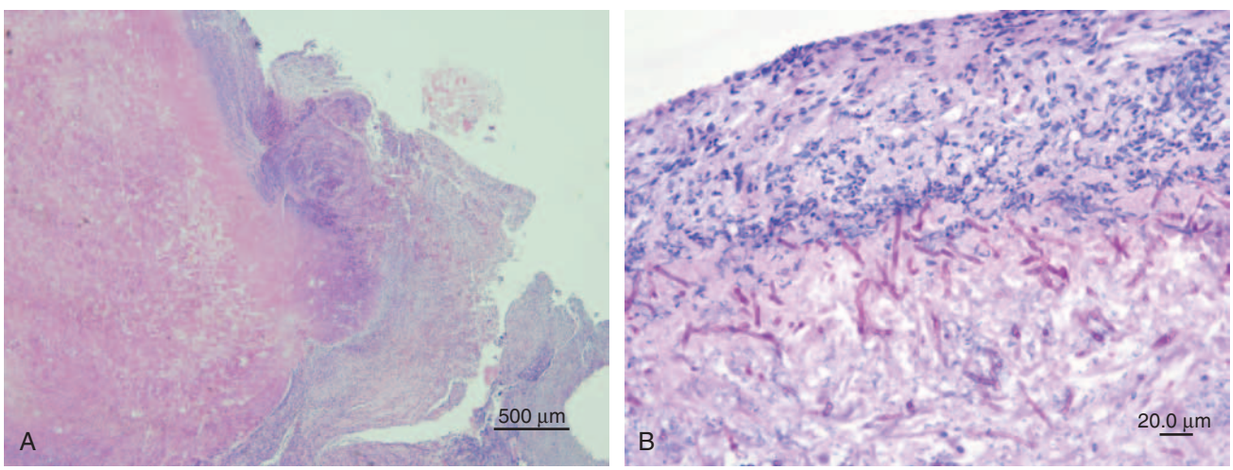

Figure 5-3 Histological examination of a retrobulbar fungal granuloma from a cat with SOA. **A,** Low magnification. **B,** High magnification. Within the necrotic center are branching, septate PAS-positive fungal filaments, surrounded by an inflammatory infiltrate of sheeting epithelioid macrophages with variable numbers of lymphocytes, plasma cells, and neutrophils, extending into a zone of peripheral fibrosis.

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Figure 5-4 The presenting complaint in this Persian cat with SNA was sneezing and unilateral right nasal discharge.

Table 5-3 Spectrum of Clinical Signs in Cats with SNA and SOA

Clinical Presentation	Clinical Signs
Sinonasal aspergillosis	Nasal discharge Discharging sinus/mass over frontal sinus or nasal bone Stertor Epistaxis
Sino-orbital aspergillosis	Exophthalmos Corneal ulceration Conjunctival hyperemia Mass or ulcer, pterygopalatine fossa Ulceration of hard palate Discharging sinus over frontal sinus Mandibular lymph node enlarged Stertor Pyrexia (temp > 39.3° C)

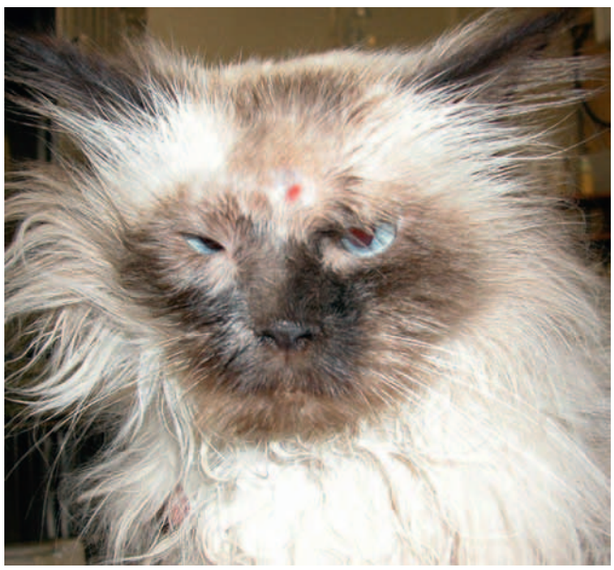

Figure 5-5 In addition to nasal discharge and sneezing, this cat with SNA had a discharging sinus over the left frontal sinus.

referable to invasive expansion of a fungal granuloma in the ventromedial orbit. These include unilateral exophthalmos with third eyelid prolapse, exposure keratitis and conjunctival hyperemia (Figure 5-6), and a mass or ulcer in the ipsilateral pterygopalatine fossa behind the last molar tooth (Figure 5-7). Stertor also is common. In the majority of affected cats, exophthalmos is unilateral, but in severe, chronic infections, bilateral exophthalmos can occur. Pain on opening the mouth typically is not present. In one cat with confirmed fungal sinusitis, marked unilateral exophthalmos resulted from retrobulbar myofascitis rather than from a fungal granuloma.[5] Invasion

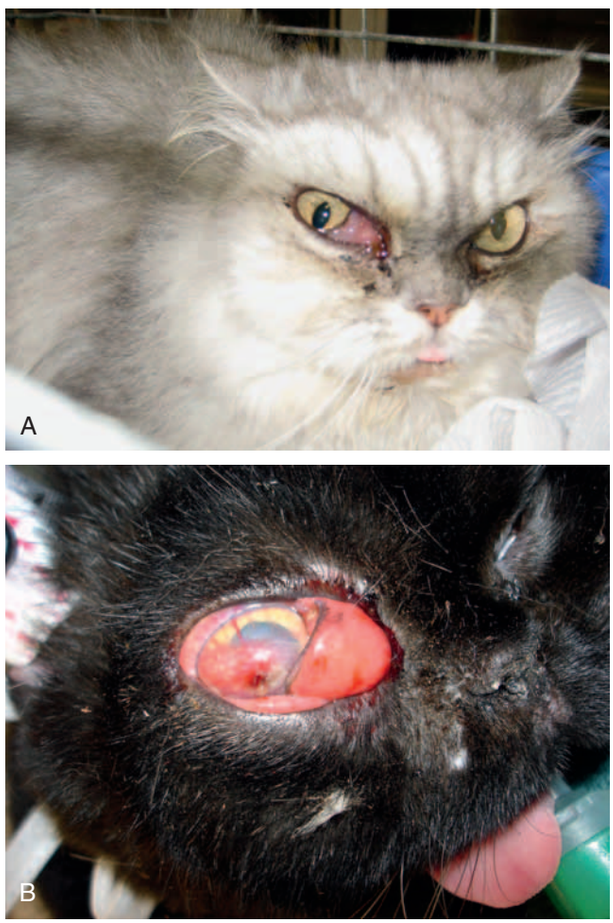

Figure 5-6 Unilateral exophthalmos with **A,** prolapse of the third eyelid, and **B,** deep corneal ulceration, in two cats with SOA.

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Figure 5-7 Three cats with SOA with a mass (**A** and **B**) or ulcer **(C)** in the pterygopalatine fossa.

through the palatine bone can cause ulceration of the hard palate (Figure 5-8).

In addition to exophthalmos, extension of infection outside the sinonasal cavity can result in facial distortion, including swelling of the nasal bridge, periorbital tissues, and soft tissues adjacent the maxilla (Figure 5-9). Neurological signs can develop subsequent to invasion of the central nervous system (CNS) through the cribriform plate or sphenoid sinus. One cat with intracranial extension of disease from the sphenoid sinus had hyperesthesia and blindness due to involvement of the optic chiasm.[11] Submandibular lymph node enlargement and pyrexia also are common at presentation (see Table 5-3). In contrast, cats with SNA usually are not pyrexic.

DIFFERENTIAL DIAGNOSIS

The differential diagnoses for URT aspergillosis depend on whether cats present with signs referable to sinonasal cavity infection or to orbital infection, or both. For cats presenting with chronic nasal discharge and sneezing, many infectious, inflammatory, neoplastic, and other causes are possible (Box 5-1). Consideration should be

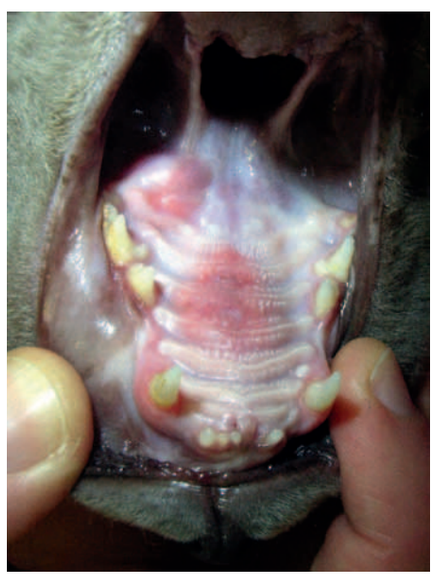

Figure 5-8 Invasion of a retrobulbar fungal granuloma through the palatine bone has resulted in ulceration of the hard palate and a mass in the left pterygopalatine fossa.

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Figure 5-9 Right exophthalmos, third eyelid prolapse, and facial distortion including swelling of the nasal bridge and maxillary soft tissues in a cat with SOA.

given to the age and breed of the cat to help rank differential diagnoses. Brachycephalic conformation should increase suspicion for SNA, although these cats also are overrepresented for viral URT infections. When epistaxis is reported, ulcerative rhinitis, neoplasia, or mycotic rhinitis are more likely. Because secondary bacterial rhinitis is common, a transient response to antimicrobial therapy does not exclude mycotic rhinitis or neoplasia. Although our experience suggests that feline URT aspergillosis is more common than thought previously, cryptococcosis remains the most commonly described cause of mycotic rhinitis. For cats with stertor and suspected nasopharyngeal mass lesions, nasopharyngeal polyps and cryptococcosis are common inflammatory causes, while lymphoma is the most common neoplastic cause.[25]

The syndrome of clinical signs present in cats with SOA could occur with other expansive infectious or neoplastic processes occurring within the orbit. Inability to retropulse the exophthalmic globe enables differentiation from buphthalmos or abnormal enlargement of the globe. The differential diagnoses for retrobulbar mass lesions are listed in Box 5-2. Direct extension of any of these processes to involve the sinonasal cavity could result in concurrent nasal discharge, sneezing, and/or epistaxis. For example, orbital lymphoma may be indistinguishable clinically from SOA (Figure 5-10).

DIAGNOSIS

Definitive diagnosis of URT aspergillosis in cats is achieved by cytological or histological identification of fungal hyphae in affected tissue, together with identification of the fungal pathogen using culture and a polyphasic taxonomic approach, as described previously (see section on classification of fungal pathogens). Obtaining a definitive

Box 5-1 Differential Diagnoses of Sinonasal Cavity and Nasopharyngeal Disease in Cats

- Neoplasia
  - Lymphoma
  - Carcinoma
  - Other
- Inflammatory
  - Nasal/nasopharyngeal polyp
  - Nasopharyngeal stenosis
- Infectious
  - Viral
    - Feline herpesvirus-1
    - Feline calicivirus
  - Mycotic nasopharyngeal diseases
    - Cryptococcosis
    - Aspergillosis
    - Phaeohyphomycosis
    - Blastomycosis
    - Histoplasmosis
    - Trichosporonosis
    - Sporotrichosis
  - Bacterial infections (primary)
    - *Mycoplasma* spp.
    - *Bordetella bronchiseptica*
    - *Chlamydophila felis*
    - *Mycobacteria* spp.
    - *Actinomyces* spp.
- Foreign body
- Congenital
  - Choanal atresia
  - Palatine defects
- Dental disease
  - Oronasal fistula
    - Advanced periodontal disease
    - Tooth root abscess
- Accumulation of excessive nasal secretions

Box 5-2 Differential Diagnoses of Feline Retrobulbar Mass Lesions

- Foreign body (e.g., grass awns)
- Abscess (odontogenic, penetrating bite wound, hematogenous)
- Orbital myofascitis (medial pterygoid muscle)
- Zygomatic salivary gland and lacrimal gland disease
- Mycotic granuloma
- Phaeohyphomycosis
- Cryptococcosis
- Aspergillosis
- Pythiosis
- Orbital pseudotumor
- Orbital fat prolapse
- Neoplasia
- Squamous cell carcinoma
- Lymphoma
- Adenocarcinoma/undifferentiated carcinoma
- Fibrosarcoma
- Melanoma
- Osteoma/osteosarcoma

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Figure 5-10 Differential diagnoses for SOA include neoplastic and infectious expansive orbital masses. This cat with right exophthalmos, purulent left nasal discharge, and an ulcerative plaque on the hard palate had orbital lymphoma.

diagnosis may require various combinations of diagnostic tests including radiography, CT, endoscopy, cytology, serology, fungal culture, and advanced mycological techniques. A stepwise diagnostic approach is recommended because there are many other differential diagnoses.

In cats with signs of sinonasal cavity disease, physical examination should include otoscopic examination to identify chronic otitis media and/or inflammatory polyps (see Chapter 30). Neurological examination is recommended because neurological sequelae such as otitis media/interna may occur with nasopharyngeal diseases and because infection can extend intracranially in cats with aspergillosis. Noninvasive investigations should be performed next, including hematology, serum biochemistries, urinalysis, serology, and thoracic radiography, to determine lower respiratory tract involvement. Retroviral serology should be performed routinely. Serological tests for aspergillosis are discussed in the next section.

A latex cryptococcal antigen agglutination test (LCAT) should be performed to exclude cryptococcal rhinitis. This test is sensitive and specific for the diagnosis of cryptococcosis. Fungal culture of superficial swabs from the rostral nasal cavity or nasal discharge is not recommended as a stand-alone test for the diagnosis of aspergillosis or cryptococcosis because these fungi may be contaminants or asymptomatic colonizers in this location.[26,27] However, isolation of *Cryptococcus* spp. in a patient with a positive LCAT or with cytological or histopathological evidence of infection is highly significant.

Bacterial culture of superficial nasal swabs rarely is rewarding due to the presence of normal flora and because bacterial rhinitis usually occurs secondary to an underlying disease. For cats with suspected viral URT infection, confirmatory tests including virus isolation, immunofluorescence, enzyme-linked immunosorbent assay (ELISA), or PCR can be performed using oropharyngeal or conjunctival swabs. When enlarged local lymph nodes or facial soft tissue masses are identified, they should be aspirated for cytological examination and culture. For cats presenting with exophthalmos, diagnostic investigation should include fluorescein staining of the cornea, retinal fundic examination, and evaluation of the orbit, in addition to investigation of the sinonasal cavity. Transocular ultrasonography is an expedient tool for confirmation of a retrobulbar mass. The next step of the investigation, incorporating diagnostic imaging, endoscopic examination of the sinonasal cavity, and biopsy collection is performed with the patient anesthetized.

RESULTS OF DIAGNOSTIC INVESTIGATIONS FOR CATS WITH URT ASPERGILLOSIS

Hematology and Serum Biochemistries

Results of complete blood counts and serum biochemistry profiles usually are unremarkable or they may reflect inflammation: for example, an inflammatory leukogram and hyperglobulinemia.[6,9,11] Establishing pretreatment renal function and baseline liver enzyme values is important, because nephrotoxicity and hepatotoxicity are common with some antifungal drugs (see treatment).

Serology

Serological tests for aspergillosis include detection of serum anti-*Aspergillus* antibodies or detection of *Aspergillus* antigen. Use of the former test has been described as an adjunct to the diagnosis of SNA in dogs. Antibodies can be detected by three methods; counterimmunoelectrophoresis (CIE), agar gel immunodiffusion (AGID), or ELISA. The AGID test, based on the principle of double diffusion, is used most commonly. In recent studies using a commercially available standardized purified aspergillin composed of *A. fumigatus, A. niger,* and *A. flavus,* the sensitivity of AGID was found to be good (67 to 77 per cent) and the specificity was excellent in dogs (97 to 100 per cent).[27,28] The ELISA has the advantage of quantification of serum immunoglobulins and, using the same antigen preparation as the AGID, has good sensitivity and excellent specificity.[28] There is little information on the diagnostic utility of serum anti-*Aspergillus* antibody detection in URT aspergillosis in cats and specificity has not been evaluated. Two of three cats with SNA tested positive for serum antibodies against *A. fumigatus* using CIE, and another cat with SNA tested positive using AGID.[3,7]

Galactomannan (GM) is a water-soluble polysaccharide cell wall component of *Aspergillus* and some other fungal species that is released in variable quantities during hyphal growth in tissues. This antigen can be detected in the serum of human patients with invasive *Aspergillus* infections using a commercially available sandwich ELISA (Platelia *Aspergillus*). GM antigen levels correlate with the tissue fungal burden and can be used to monitor clinical outcome and response to therapy. Rising GM levels are often associated with treatment failures in human

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patients.[29] There are limitations to the use of the GM ELISA in patients with invasive aspergillosis. For example, false-positive results can occur following treatment with ß-lactam antibiotics due to contamination with cell wall components of *Penicillium* spp. during drug production. The sensitivity of the GM ELISA varies considerably with immune status. In profoundly immunocompromised patients, sensitivity is greater than 90 per cent, while in less immunocompromised patients, sensitivity may be as low as 30 per cent. This is partly due to a lower fungal burden in the latter group. Further, concurrent antifungal therapy may delay detection of GM and result in lower GM levels due to decreased fungal load.[29] This same commercially available ELISA has been evaluated in dogs and was found to be unreliable for the diagnosis of SNA.[28] A GM optical density (OD) index of greater than 0.5 was considered to be positive. This is the accepted standard for diagnosis of invasive aspergillosis in human beings and the standard recommended by the manufacturer. Test results were positive in 24 per cent of dogs with SNA, 11 per cent of dogs with nasal tumors, 9 per cent of dogs with lymphoplasmacytic rhinitis, and 24 per cent of control dogs.[28] Given that SNA in dogs is noninvasive, the low sensitivity is hardly surprising. Reasons for poor specificity were not identified; concomitant use of ß-lactam antimicrobials was found to have a negligible influence on results in dogs.

The use of GM assays for diagnosis and monitoring of feline aspergillosis has not been evaluated. GM was measured in six of our culture-positive cases using the Platelia *Aspergillus* assay. Using a cut-off value of 0.5, three cats with SOA were negative (GM OD index 0.11 to 0.34), one cat with SNA was positive at 20.98, and two cats with SOA were positive at 18.22 and 3.82. In the latter case, in which combination antifungal treatment using amphotericin B, posaconazole, and terbinafine failed, the GM OD index increased subsequently to 14.83. Further studies are underway to investigate the use of GM assay in diagnosis and monitoring of therapy in cats with URT aspergillosis.

Diagnostic Imaging—Radiography, CT, and Ultrasonography

Imaging studies should be performed prior to more invasive diagnostic procedures including rhinoscopy and biopsy because any resultant inflammation or hemorrhage may obscure subtle lesions and induce imaging abnormalities. For cats presenting with exophthalmos, transocular ultrasonography is recommended to identify retrobulbar mass lesions (Figure 5-11). For further investigation of suspected sinonasal cavity or sino-orbital disease, CT is the diagnostic imaging modality of choice. Both CT and magnetic resonance imaging (MRI) have the advantage over conventional radiography of cross-sectional imaging. Other advantages of CT over conventional radiography demonstrated in dogs with SNA include increased diagnostic sensitivity, ability to adjust the contrast scale to optimize optical density and discriminate fine turbinate structures, and better evaluation of the cribriform plate using multiplanar reconstructions.[30,31] Computed tomography generally is superior to MRI for the evaluation of destructive lesions in bony

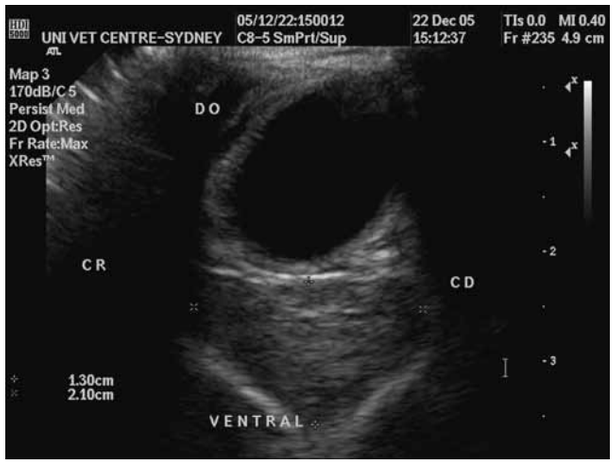

Figure 5-11 Transocular ultrasound of a cat with a retrobulbar fungal granuloma, 10 MHz linear probe.

structures contiguous with the sinonasal cavity. In cats with suspected intracranial extension of infection, MRI after intravenous contrast administration is superior to CT for evaluation of intracranial soft-tissues.

Regardless of which imaging modality is used, the nasal cavity, nasopharynx, frontal sinuses, and tympanic bullae should be evaluated routinely. In patients with nasopharyngeal mass lesions, occlusion of the auditory tubes can result in secondary bullous effusion, which may be asymptomatic.[32] If radiography is used, views should include lateral skull, ventrodorsal skull, dorsoventral occlusal view or open-mouth ventrodorsal view, and rostrocaudal open-mouth or rostral 10° ventrocaudodorsal oblique views. Findings on radiography in cats with SNA and SOA include destructive rhinitis, lysis of paranasal bones, and focal increased soft tissue opacities.[3,7]

We have performed CT on 10 cats with URT aspergillosis, including three with SNA and seven with SOA. Our findings are summarized in Table 5-4. The most common findings were punctate lysis of the orbital lamina (see Figure 5-2), opacification of the sphenoid and frontal sinuses due to fluid or soft tissue (Figure 5-12), and a soft tissue mass in the choanae or nasopharynx. Eight of the 10 cases had punctate lysis of the orbital lamina, including two of three cases of SNA. Of the seven cases of SOA, six had an orbital mass, and in one case the CT was performed after exenteration of the orbit. There was irregular enhancement of the orbital mass in all cases after intravenous contrast administration (Figure 5-13). Orbital masses were present in the ventromedial aspect of the orbit, causing lateral and dorsal displacement of the globe. In five cats with SOA, there was a mass effect involving the soft tissues adjacent the maxilla, and four cats had soft tissue palatine masses. Punctate lysis of the cribriform plate was seen in two cases, one cat with SNA and one with SOA (Figure 5-14). Thickening of the mucosa adjacent to the inner surface of the bones of the frontal sinus, a typical finding in canine SNA, was seen in three cats, one with SNA and two with SOA (Figure

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Table 5-4 Computed Tomographic Findings in 10 Cases of Feline URT Aspergillosis

Structure	Lysis	Sclerosis	Soft Tissue or Fluid Density	Thickened Mucosa	Soft Tissue Mass
Turbinates	5		3	4	
Maxilla	4				5
Palate	3				4
Cribriform plate	2				
Orbital lamina	8				
Frontal sinus and frontal bone		2	7	3	
Sphenoid sinus			7		
Septum	2				
Cribriform plate	2				
Nasopharynx/choanae					7
Orbit			1		6

Data from three cats with SNA and seven with SOA. In one case, CT was performed after exenteration of the globe.[11] Sinonasal abnormalities were unilateral in three cases and bilateral in seven cases.

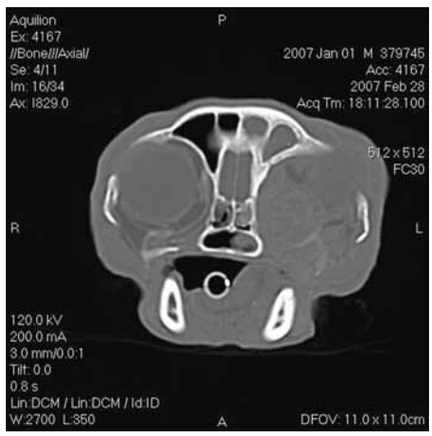

Figure 5-12 Transverse CT of skull. Opacification of the sphenoid and left frontal sinuses and nasopharyngeal mass in a cat with an expansive left orbital fungal granuloma.

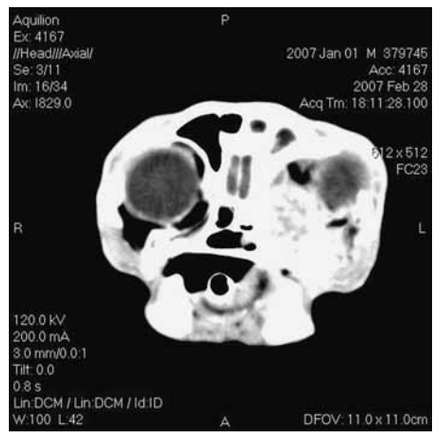

Figure 5-13 Postcontrast CT of cat in figure 5-12 showing irregular enhancement of the left orbital mass.

5-15).[30,31] There was evidence of unilateral sinonasal cavity involvement in three cases and of bilateral disease in seven affected cats. The tympanic bullae were normal in all cases. Computed tomographic findings in previously reported cases (six cats with SNA and three cats with SOA) were similar, except for one case of SNA in whom a free, irregular calcified mass was present in the cranial part of the right nasal cavity.[5-9] On histological examination, the mass was identified as calcified necrotic tissue containing fungal hyphae. Concretions formed from deposition of calcium oxalate or phosphate crystals within tissues occur in human patients with sinus aspergilloma.[33]

Computed tomographic findings in cats with SOA are similar to those in human beings with chronic invasive fungal sinusitis and chronic granulomatous invasive

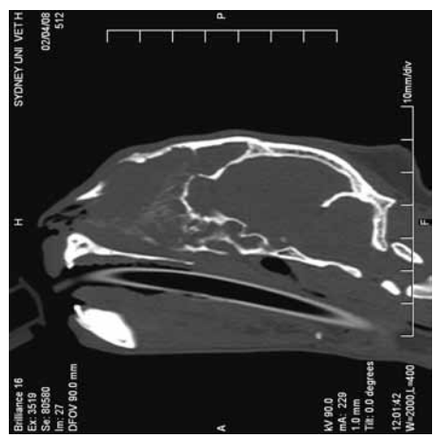

Figure 5-14 Sagittal CT skull. Punctate lysis of the cribriform plate and lysis of the nasal bone dorsally, in a cat with severe SNA.

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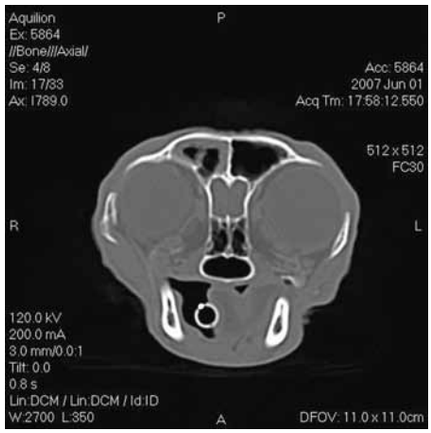

Figure 5-15 Transverse CT skull. Thickening of the mucosa adjacent to the inner surface of the bone of the left frontal sinus in a cat with SNA.

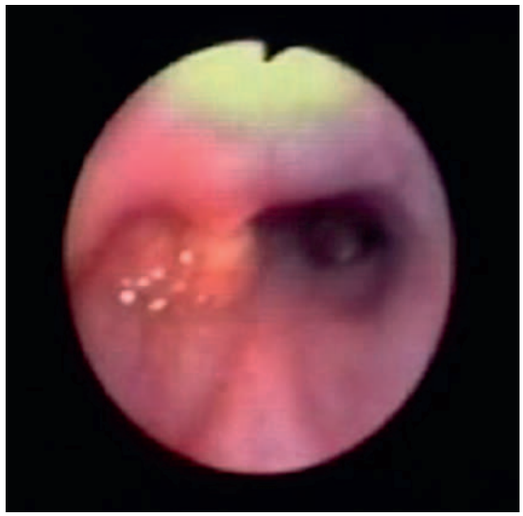

Figure 5-16 Choanal mass in a cat with SOA visualized using a retroflexed pediatric endoscope, before biopsy.

fungal sinusitis.[34] On noncontrast CT in human patients, a hyperattenuating soft tissue collection, sometimes masslike, within one or more paranasal sinuses is typical. There may be destruction of sinus walls and extension into adjacent tissues. In both human beings and cats with URT aspergillosis, differentiation of invasive mycotic disease from malignant neoplasia may not be possible from imaging findings alone. On CT, destruction of maxillary turbinates is seen commonly in cats with inflammatory or neoplastic sinonasal disease.[35] Severe destruction of both maxillary turbinates and ethmoturbinates, particularly when unilateral, and/or lysis of paranasal bones have been considered more predictive of sinonasal neoplasia than of inflammatory disease.[35] However, there were no cases of mycotic rhinitis in the inflammatory disease group in that study. Many findings that were significantly associated with neoplasia are common in cats with URT aspergillosis, including unilateral lysis of ethmoturbinates, unilateral lysis of the maxilla, lysis of the vomer bone, lysis of the orbital lamina, and unilateral soft tissue or fluid within the sphenoid sinus, frontal sinus, and/or retrobulbar space.[35]

Collection of Diagnostic Biopsies

Nasopharyngoscopy and rhinoscopy are performed next to visualize the nasopharynx and nasal cavity and to obtain biopsy specimens for cytological and histological examination and culture. We freeze additional biopsy specimens routinely at this time. These samples can be retrieved for PCR if fungal hyphae are seen in tissues but fungal culture is negative. Because many cats with both SNA and SOA have granulomatous mass lesions containing fungal hyphae within the choanae or nasopharynx, retroflexed endoscopy using a pediatric bronchoscope often is rewarding (Figure 5-16). Masses in this location can be biopsied using endoscopic biopsy forceps. During nasopharyngeal endoscopy, each ventral nasal meatus is catheterized via the naris with a urinary catheter (size 5 or 6 French gauge) to check patency and to help dislodge foreign material or masses. Regional anesthesia of the pharynx with 2 per cent topical lidocaine gel and maxillary nerve blocks using 0.5 per cent bupivacaine facilitates a decreased plane of general anesthesia in some cats during nasopharyngeal endoscopy.

The rostral nasal cavity can be evaluated using rigid rhinoscopy to visualize turbinate destruction and fungal plaques. Rhinoscopes with constant irrigation systems that enable biopsies to be taken during direct examination are preferred because fungal plaques could be missed during blind collection.

Nasal cavity lavage may yield larger biopsy specimens than can be acquired endoscopically. Vigorous saline flushing from the nasal cavities into the nasopharynx is useful to dislodge foreign bodies and diagnostic fragments of friable tumors such as lymphoma or granulomas. A cuffed endotracheal tube should be used. The pharynx is packed with sterile swabs to trap flushed material. A 10-mL aliquot of sterile saline is flushed briskly through one naris into the ventral nasal meatus while occluding the other naris. The procedure is repeated several times on each side. Culture of lavage fluid usually returns nonspecific results.

For cats with SOA, fine-needle biopsies of retrobulbar masses can be obtained under ultrasound guidance or via the oral cavity. Computed tomography–guided biopsies and aspirates also have been described.[9] Computed tomography is useful to identify involvement of paranasal subcutaneous soft tissues and sinuses. The former can be biopsied surgically, and the latter can be accessed via sinus trephination. When sinus involvement is identified and diagnostic biopsies can not be collected during rhinoscopy or nasopharyngeal endoscopy, sinus trephination using a Jacob chuck and intramedullary pin (3.2 to 4 mm diameter) enables endoscopic examination of the lateral compartment of the frontal sinuses and ready access to material for culture, histology, and PCR. This procedure should be performed with caution in brachycephalic cats because they have shallow sinuses in close proximity to the cranial vault.

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TREATMENT AND PROGNOSIS

ANTIFUNGAL SUSCEPTIBILITY TESTING

The typical antifungal susceptibility profile of *Neosartorya* spp. isolated from cats with aspergillosis includes susceptibility to amphotericin B and posaconazole, intermediate or dose-dependent susceptibility to itraconazole and voriconazole, and resistance to 5-flucytosine, ketoconazole, and fluconazole.[11] In contrast, both voriconazole and posaconazole typically have greater activity than itraconazole in vitro against both *A. fumigatus* and other *Aspergillus* spp.[36] Interestingly, elevated minimum inhibitory concentrations (MICs) of voriconazole to *Neosartorya* spp. have been documented elsewhere.[13] However, the correlation between in vitro antifungal susceptibility test results and in vivo response to therapy is only modest at best. Other factors that influence antifungal activity in vivo include drug pharmacokinetics (e.g., stability, metabolism, drug interactions, protein binding, metabolites, tissue penetration), host factors (e.g., immune status, underlying disease), site of infection (e.g., presence of foreign body, source of infection), and virulence factors of the fungal pathogen.[37] Further, in vitro results are influenced by technical factors including the concentration of the fungal inoculum, the composition and pH of the medium, incubation temperature, and duration of incubation.

SYSTEMIC ANTIFUNGAL THERAPY

For treatment of invasive aspergillosis in human beings, voriconazole has replaced amphotericin B as the drug of choice for first-line therapy. Posaconazole is used for antifungal prophylaxis in high-risk patients and for salvage therapy. The echinocandins are reserved for salvage therapy or combination antifungal therapy for refractory invasive aspergillosis.[38] Combination antifungal therapy may provide enhanced efficacy compared to single-drug therapy for treatment of invasive aspergillosis. This concept has been controversial because in previous studies, when mice with invasive aspergillosis were treated with ketoconazole prior to amphotericin B, there was a marked decrease in the efficacy of the latter drug. It was proposed that the azole blocked the synthesis of the ergosterol target necessary for the binding of amphotericin B. Antagonism between itraconazole and amphotericin B also has been demonstrated in vitro against isolates of *A. fumigatus*.[39] However, more recently treatment of murine models of invasive aspergillosis with a triazole antifungal (voriconazole or posaconazole) and amphotericin B was not antagonistic, and treatment with liposomal amphotericin B and voriconazole in combination was significantly superior to monotherapy with either drug.[40,41]

Triazole Antifungals

Triazole antifungals, which have three nitrogen molecules in the azole ring, include fluconazole, itraconazole, voriconazole, and posaconazole. By binding to lanosterol-14α-demethylase, triazoles inhibit the synthesis of ergosterol, an essential lipid component of fungal cell walls. Fluconazole has low activity against filamentous fungi and is not recommended for treatment of aspergillosis.[42] Itraconazole has fungistatic activity against *Aspergillus,* and its pharmacological disposition has been studied in cats.[43] It is metabolized by the liver and distributed widely in body tissues other than the CNS. Oral bioavailability can be variable and is highest for capsules when administered with food and for oral solution when administered on an empty stomach. The oral solution is absorbed more effectively than capsules in cats, such that dose reduction may be necessary. Adverse effects including gastrointestinal signs and hepatotoxicity are not uncommon, are usually dose related, and resolve on cessation of therapy (Table 5-5). Voriconazole (structurally similar to fluconazole) and posaconazole (structurally similar to itraconazole) were developed as more efficacious agents for treating filamentous fungal infections and to improve on the absorption, tolerability, and drug interaction profile of itraconazole. Both voriconazole and posaconazole are fungicidal against *Aspergillus*. Their pharmacokinetics have been studied in human beings and dogs but not in cats at the time of writing.[44,45] Posaconazole is available only as a suspension for oral use. Its absorption is increased with food, especially high-fat meals, and is optimal in human patients when given as four daily divided doses. Posaconazole is metabolized in the liver by glucuronidation and is excreted primarily in bile and urine. Common adverse effects include headaches and gastrointestinal signs such as nausea, diarrhea, and vomiting. Hepatoxicity is uncommon.[42]

Posaconazole was used in the treatment of 10 cats with URT aspergillosis (see Table 5-5).[11] Inappetence and a twofold elevation in alkaline phosphatase (ALP) were seen in one cat, both of which resolved when the drug dosage was decreased. Posaconazole was well tolerated in the other nine cats and no adverse effects were recorded. Liver enzymes were within reference ranges during routine serum biochemical monitoring, which was performed at least twice in all cats. There are two other case reports describing the clinical use of posaconazole in cats for treatment of SOA and infection of the subcutis of the nose by *Mucor* species respectively. Posaconazole was well tolerated in both cases.[9,46] Voriconazole, in contrast, has 95 per cent bioavailability after oral administration in the absence of food. It is metabolized in the liver by several cytochrome P450 enzymes. In human beings, adverse effects are more common with voriconazole than with posaconazole and include visual disturbances and hepatotoxicity. Blurred vision, photophobia, and other visual changes occur in up to one third of patients treated with either IV or oral formulations. These changes often resolve in a few weeks even if therapy is continued.[42] There are no published reports of the use of oral voriconazole in cats. The authors have treated three cats with URT aspergillosis using voriconazole, two of whom showed adverse effects that resolved after drug discontinuation. One cat who had received 12 mg/kg (50 mg) daily for four doses developed anorexia and neurological

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Table 5-5 Drugs Used for Treatment of Aspergillosis in Cats

Drug and Formulation	Dosage and Route of Administration	Adverse Effects
Itraconazole 100 mg capsules 10 mg/mL oral suspension (Sporanox; Janssen)	5 mg/kg PO q12h or 10 mg/kg PO q24h	Gastrointestinal—anorexia, vomiting Hepatotoxicity—increased liver enzyme activity, jaundice. Monitor ALP/ALT monthly. If hepatoxicity occurs, reduce dose to 5 mg/kg PO q24h or 10 mg/kg PO q48h
Posaconazole 40 mg/mL liquid (Noxafil, Schering-Plough)	2.5-3.75 mg/kg PO q12h	Hepatotoxicity
Voriconazole 50 mg tablets 40 mg/mL powder for oral suspension (Vfend, Pfizer)	5 mg/kg PO q24h	Neurotoxicity—blindness, ataxia, dazed.
Terbinafine 250 mg tablets (Lamisil, Novartis)	30 mg/kg PO q24h	Gastrointestinal—anorexia, vomiting, diarrhea
Amphotericin B deoxycholate 50 mg vial (Fungizone, Bristol-Myers)	0.5 mg/kg of 5 mg/mL stock solution in 350 mL/cat of 0.45% NaCl + 2.5% dextrose SQ 2-3 × weekly to a cumulative dose of 10-15 mg/kg.	Nephrotoxicity—pretreatment of stock solution by heating to 60° C for 5 minutes reduces nephrotoxicity. Monitor urea/creatinine every 2 weeks. Discontinue for 2-3 weeks if azotemia.
Liposomal Amphotericin (AmBisome, Gilead)	1-1.5 mg/kg IV q48h to a cumulative dose of 12-15 mg/kg. Given as a 1-2 mg/mL solution in 5% dextrose by IV infusion over 1-2 h	Azotemia.

signs including altered mental status, dilated pupils, and hind limb ataxia. Another cat treated with 5 mg/kg daily developed behavioral changes and apparent visual impairment. A third cat was treated with 6 mg/kg daily (25 mg) for 10 days with no adverse effects.

Polyenes

Amphotericin B is a polyene macrolide antibiotic derived from the actinomycete *Streptomyces nodosus* with fungicidal activity against *Aspergillus*. Preferential binding to ergosterol in the fungal cell membrane results in altered permeability, leakage of cell components, and cell death. Amphotericin B deoxycholate must be administered parenterally, either intravenously or as a subcutaneous infusion (see Table 5-5). The major toxicity in all species is nephrotoxicity. The development of lipid formulations of amphotericin B, which are less toxic than amphotericin B deoxycholate (AMB-D), has resulted in enhanced antifungal activity because increased doses of amphotericin B can be administered with improved delivery of active drug to sites of infection. Lipid formulations of amphotericin B are concentrated in the reticuloendothelial system, and liposomal amphotericin B attains high concentrations in brain tissue. The authors have used liposomal amphotericin B (AMB-L) in cats with aspergillosis at a dosage of 1 to 1.5 mg/kg IV q48h (see Table 5-5). Although AMB-L is much less nephrotoxic than AMB-D, nephrotoxicity still can occur, and renal function should be monitored during the treatment period. AMB-D has been administered directly into the retrobulbar space in isolated cases of invasive SOA in human beings as an adjunct to surgical debridement when maximal drug doses have been given systemically.[47] In vitro and in vivo resistance to amphotericin B has been reported in one case of feline SOA.[9] In vitro resistance to amphotericin B (MIC > 2 mg/L) is rare and arises because of a mutation in the ergosterol biosynthesis pathway resulting in production of ergosterol-like compounds that have reduced binding affinity for amphotericin B.[48]

Terbinafine

Terbinafine is an allylamine antifungal. It inhibits fungal ergosterol synthesis via inhibition of the enzyme squalene mono-oxygenase, which converts squalene to ergosterol. Synergy between antifungal triazoles and terbinafine has been demonstrated against filamentous fungi in vitro.[49] To date, the main indication for terbinafine in cats has been in the treatment of dermatophytosis.[50]

Echinocandins

The echinocandins, including caspofungin, micafungin, and anidulafungin, inhibit 1,3-ß-D-glucan synthesis, an essential polysaccharide cell wall component of many fungal species. The echinocandins are the most recently developed class of antifungal drug and are characterized by low toxicity and rapid fungicidal activity. They have additive or synergistic activity with amphotericin B and triazoles against filamentous fungi, including *Aspergillus*.[51] Because the oral bioavailability of echinocandins is very low, they must be administered parenterally. In human beings, they are eliminated primarily as metabolites in the urine and in the feces following degradation

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in the liver via nonoxidative pathways. Metabolism is thought to be independent of the cytochrome system and does not inhibit cytochrome P450 isoenzymes. The pharmacology and pharmacokinetics of echinocandins have not been studied in cats, and there are no reports of their use in this species.

THERAPEUTIC APPROACH TO SNA AND SOA IN CATS

Because of its more invasive nature, URT aspergillosis in cats is more difficult to treat than SNA in dogs. In general, treatment carries a better prognosis for SNA than SOA. The prognosis for resolution of SOA is poor.

SNA—Topical Clotrimazole Infusions

The technique of single topical sinonasal clotrimazole infusions, which has good efficacy for treatment of SNA in dogs, has been reported in three cats.[6-8] In one cat, infection was confined to the sinonasal cavity and treatment with oral itraconazole and a 1 per cent clotrimazole infusion was successful.[7] In two other cats with SOA, a clotrimazole infusion was part of a multimodal therapeutic approach. One cat was euthanized because of suspected progression of infection despite treatment with itraconazole and radical surgical debridement of the orbit, including exenteration of the globe.[6] In another cat with SOA, treatment with itraconazole, topical clotrimazole, and AMB-D did not resolve infection. However, the cat was cured after treatment with oral posaconazole administered for 16 weeks.[9] Our current recommendation is to restrict the use of topical 1 per cent clotrimazole infusions to the treatment of cats with SNA in whom there is an intact cribriform plate, no evidence of orbital involvement on CT, and no histological evidence of submucosal fungal invasion. Commercial preparations of 1 per cent clotrimazole in a polyethylene glycol base are available. Formulations that contain propylene glycol or isopropyl alcohol should be avoided because of greater potential for pharyngeal edema, as reported in dogs.[52] Concomitant systemic antifungal therapy with itraconazole or posaconazole also is recommended.

SNA—Systemic Antifungal Therapy

Antifungal triazole drugs are the mainstay of therapy and can be given alone or in combination with terbinafine or amphotericin B (see Table 5-5). Itraconazole or posaconazole are the triazoles of choice. When concurrent topical azole therapy is administered (i.e., topical clotrimazole infusion) and disease is mild, amphotericin B administration may not be necessary. Also, it should be noted that treatment with itraconazole alone has been successful occasionally in resolving SNA.[8] For cases of severe SNA where there is evidence of lysis of the orbital lamina or cribriform plate and risk of extension of infection beyond the sinonasal cavity, we recommend concurrent administration of amphotericin B (see Table 5-5). When the course of amphotericin B is completed, therapy can be continued with the oral triazole and terbinafine. The duration of treatment necessary to resolve SNA varies depending on the severity of infection, but usually is 4 to 6 months.[7]

SOA—Systemic Antifungal Therapy

The optimal treatment regimen for SOA in cats has not been identified. Of 20 reported cases, treatment was attempted in 14 and infection resolved in only four patients.[9,11] Of the cases treated successfully, three were treated with medical therapy alone, including one that resolved after posaconazole administration for 16 weeks.[9] Infection resolved in one cat treated for 7 months with combination therapy using itraconazole, AMB-D (cumulative dose 11 mg/kg), and terbinafine. A third cat responded well to 8 months of therapy with ICZ and AMB-D (cumulative dose 14 mg/kg). Exophthalmos improved dramatically in this patient after 7 days of treatment with itraconazole and AMB-D (cumulative dose 14 mg/kg). However, clinical and CT evidence of infection recurred 3 weeks after itraconazole was stopped and after 8 months of treatment. Treatment was reinstituted with combination therapy using terbinafine and posaconazole for 4 months, followed by posaconazole alone for a further 3 months. Signs resolved and the cat remained asymptomatic 1 year later. The third case was treated with surgical debridement of the frontal sinuses during diagnostic investigation followed by AMB-D (cumulative dose 10 mg/kg) and itraconazole for 7 months. Clinical signs of infection resolved and the cat was asymptomatic 2 years later. Treatment failures occurred in four cats who were treated with medical therapy alone, and in six patients treated with combination antifungal medical therapy and radical surgical debridement of orbital granulomas, including exenteration in five cases.[11]

It is clear that the factors that determine whether a cat with SOA will respond to treatment have yet to be identified. Consideration should be given to surgical debridement of large fungal granulomas within the retrobulbar space, although evidence for efficacy of this approach in cats currently is lacking. Treatment of immunocompetent humans with chronic granulomatous sinusitis involves aggressive surgical debridement and chronic antifungal therapy.[52] Current recommendations for medical therapy in cats with SOA are the same as for cats with severe SNA (see Table 5-5).

SUMMARY

URT aspergillosis can cause a spectrum of disease in cats ranging from noninvasive sinonasal cavity infection to severe invasive sino-orbital disease. *Aspergillus fumigatus* is the cause of some infections, but a closely related species, *Neosartorya* is implicated more commonly. Identification of *Neosartorya* infection requires a polyphasic taxonomic approach, including fungal colony cultural characteristics and PCR. Aspergillosis should be included in the differential diagnosis of retrobulbar disease, and diagnostic investigations should include imaging of the sinonasal cavity. More work needs to be done to identify the optimal therapeutic approach to treatment of upper respiratory aspergillosis in cats.

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