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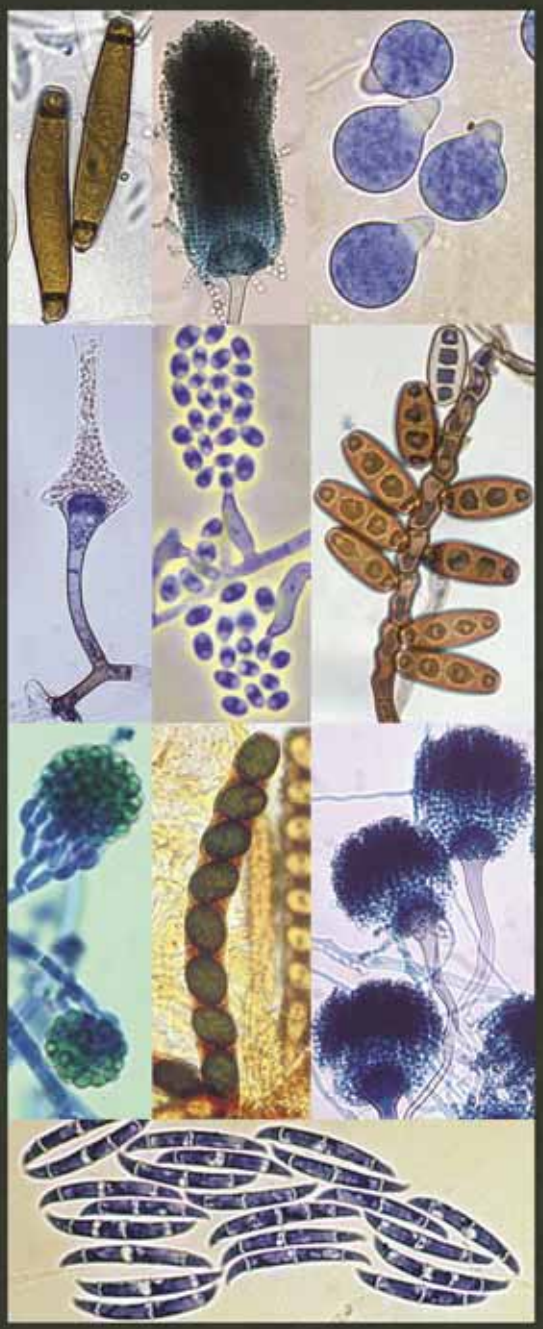

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Contents

Cover image: Cryptococcus gattii *india ink plus janus green mount of a CFS specimen with the cryptococci arranged as the 'Southern Cross' and a montage showing microscopic morphology of* Exserohilum rostrum, Aspergillus lentulus, Conidiobolus coronatus, Saksenaea vasiformis, Phialophora *sp.,* Bipolaris australiensis, Gliiocladium sp., Sordaria fumicola, Aspergillus fumigatus, Fusarium solani. *Images courtesy of David Ellis, University of Adelaide.*

Medical and veterinary mycology



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Fungi, the second most frequent eukaryotic organisms[1], are responsible for many diseases in plants, animals and humans. They cause significant problems and economic losses in agriculture, food security and the health system as well as having an impact of the biodiversity and ecosystems[2]. Of the estimated 5 million fungal species[1], about 600 are known to cause human or animal infections, ranging from superficial infections of the nails and skin caused by dermatophytes, through mucocutaneous candidiasis to life-threatening invasive fungal infections caused by *Candida*, *Cryptococcus* and *Aspergillus* species, among others[3]. The number of reported infections have significantly increased over the past three decades, with serious impacts on public health and an increased risk of biodiversity loss among animal species[2,4]. In humans, superficial infections affect an estimated 1.7 billion (25%) individuals worldwide. The majority of superficial infections are not lethal but they can cause serious discomfort. Oropharyngeal or genital mucosal infections are also common and can be disabling. An estimated 75% of the women of childbearing age suffer from vulvovaginitis, mainly caused by *Candida* species[5], which are the third most common opportunistic fungal disease agents after *Aspergillus* spp., worldwide[4]. Invasive fungal diseases are less common but they cause significant morbidity and mortality, killing about 1.5 million people every year worldwide[4], and are associated with substantial healthcare costs[6]. More than 90% of fungus-related deaths are caused by four fungal genera: *Aspergillus, Candida, Cryptococcus* and *Pneumocystis*[4,7–9]. Fungi are also responsible for other debilitating diseases, including blindness, and chronic subcutaneous infections. The growing threat of fungal infections is reflected in the global market for antifungals, which is expected to grow from $11.8 billion in 2013 to $13.9 in 2018[9].

Most fungal species that cause infections are opportunistic, with a minority considered as primary pathogens of humans or animals. As a result, understanding the pathogenesis of fungal infections is critical to improve antifungal treatment of confirmed disease. The application of new molecular and biochemical techniques has largely contributed to the development of improved fungal identification and new antifungal therapies. However, development of better and safer antifungal drugs is still needed.

Despite the growing importance of fungal infections, the number of fungal studies (private or public) and the available research funding lag significantly behind that of bacteria and viruses. To overcome this it is important to raise public awareness of fungal infection and their consequences.

In May 2015, the Australian New Zealand Mycology Interest Group (ANZMIG) of the Australian Society of Infectious Diseases (ASID) is hosting the 19th Congress of the International Society of Human and Animal Mycology (ISHAM) and will welcome medical mycologists and infectious diseases clinicians from around the globe. Discussions will include the latest findings in basic, applied and clinical medical mycology.

Australia has an active medical and veterinary mycological community that has made many pivotal contributions to medical and veterinary mycology at the international level. Historical studies focused principally on dermatophytes especially those associated with Australian Aborigines and native animals. Today international collaborations range from basic science projects studying DNA barcoding of pathogenic fungi by using comparative genomics to develop new standardised diagnostic tools[10], and discovering

molecular mechanisms of fungal pathogenicity and their application to clinical studies[11–13].

Cryptococcosis has been an important endemic fungal infection of both humans and animals in Australia since first being recognised in the early 1900s. However, it was the discovery of the natural habitat of *C. gattii* in 1990[14] that provided a major impetus for research on the epidemiology and ecology of this fungus. These studies defined the epidemiology of these serious fungal infections in Australia[15] and elsewhere and through global collaborations, have revealed the origin of highly pathogenic strains and increased understanding of the molecular basis of cryptococcal pathogenicity[16,17].

The Australian and New Zealand Mycoses Interest Group (ANZMIG) is now the region's premier medical mycology forum. The role of ANZMIG is to foster and promote research, education and training in the pathogenesis, microbiology, epidemiology, diagnosis, treatment and prevention of human infections caused by fungi and closely related organisms. To achieve this, ANZMIG has been instrumental in the conception, design and implementation of many mycological research and educational projects. These include; the Australian Candidemia Study[11] and associated studies on invasive fungal infections in haematology patients and candidemia in ICU patients; the Australian *Scedosporium* study[12], the *Aspergillus* haematology study and several *Cryptococcus* studies[13]. ANZMIG has also been instrumental in publishing the Australian Guidelines for use of antifungal agents in treatment of invasive fungal infection[18]. It hosts a biannual Mycology MasterClass, which will be offered concurrently with the 19th ISHAM congress is 2015, as well as many other clinical mycology symposia and conference programs.

Australian researchers are active in the detection and identification of mycological disease from a wide variety of sources but especially human community- and hospital-acquired infections. The medical mycology community collaborates together through a well-established national network, the sharing of materials, the distribution of materials for quality control diagnostics and a variety of other interactions.

The Australian Society of Microbiology is pleased to showcase Australian mycological research to the world by devoting this special issue of Microbiology Australia to the ISHAM conference. Australia has been and is a driving force in the advancement of human and veterinary mycology, including clinical trials, development of antifungal guidelines, molecular epidemiology, and basic genetic studies of fungal pathogenesis. With our colleagues from around the world, we trust that ISHAM in Melbourne will lead to, the expansion of existing global collaborations and the establishment of new ones between medical mycologists, infectious disease clinicians and basic researchers.

We wish all delegates of the 19th ISHAM congress an enjoyable and stimulating time in Melbourne.

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Biographies

The biography for **Professor Wieland Meyer** is on page 48.

The biography for **Laszlo Irinyi** is on page 48.

Professor Tania Sorrell is an Infectious Diseases physician and academic with long-standing research interests in the pathogenesis of cryptococcal infections, the epidemiology and management of systemic yeast infections and development of new fungal diagnostics and antifungal therapeutics. She is a past President of the Australasian Society for Infectious Diseases (ASID), a current member of the international Mycoses Study Group Education and Research Steering Committee and the Mycoses Interest group of ASID. She is Director of the Marie Bashir Institute for Infectious Diseases and Biosecurity at the University of Sydney and Service Director of Infectious Diseases and Sexual Health, Western Sydney Local Health District.

DNA barcoding of human and animal pathogenic fungi: the ISHAM-ITS database

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Human and animal fungal pathogens are a growing threat worldwide. They lead to emerging infections and create new risks for established ones. As such, there is a growing need for the rapid and accurate identification of mycoses agents to enable early diagnosis and targeted antifungal therapy. An international consortium of medical mycology laboratories was formed in order to establish a quality controlled ITS database under the umbrella of the ISHAM (International Society for Human and Animal Mycology) working group on 'DNA barcoding of human and animal pathogenic fungi'. The new database provides the medical community with a freely accessible tool via http://www.isham.org/ or directly at http://its.mycologylab.org/ to rapidly and reliably identify most mycoses agents. The average intra-species variation of the ITS sequences currently included in the database ranges from 0 to 2.25%, highlighting the fact, that the ITS region on its own is insufficient for the reliable identification of certain pathogenic fungal species.

The number of human and animal mycoses, ranging from superficial to invasive fungal infections, has significantly increased over the past three decades, causing serious public health burdens and increased risk of biodiversity loss among animal species[1,2]. To better understand, control and treat fungal infections, more rapid and accurate identification of the causal agents is essential. Traditional identification based on morphology and biochemistry is time-consuming and requires morphological and taxonomical expertise. To overcome these limitations, DNA barcoding offers a practical approach for species identification, which is less demanding in terms of taxonomical expertise. DNA barcoding consists of using short sequences (500–800 bp) for the identification of organisms at species level by comparison to a reference collection of well-identified species. The concept of barcoding is that species identification must be accurate, fast, cost-effective, culture independent, universally accessible and feasible for non-experts. The principles of barcoding are that: (i) interspecies variation should exceed intraspecies variation, to create a barcode gap[3], and (ii) identification is straightforward, when a sequence is unique to a single species and constant within each species[4].

ITS as the current official DNA barcode for fungi

The current official fungal DNA barcode[5], the internal transcribed spacer (ITS) region has long been used in molecular identification and phylogenetic studies of human and animal pathogenic fungi. The ITS region is easy to amplify with universal primers suitable for most fungal species and show sufficient genetic variability at interspecies level. The ITS sequences are used routinely by the medical community for fungal identification at the species level on the basis of matching sequences in publicly accessible databases, such as GenBank. However, its wide-spread applicability is still limited by the absence of quality-controlled reference databases. According to a recent study, 10% of the publicly available fungal ITS sequences were identified incorrectly at species level. Many of the ITS sequences deposited in public databases are incomplete or wrongly annotated[6]. Moreover, no studies have been done to evaluate the ITS region as an official standard barcode in clinically relevant fungal species.

Establishment of the ISHAM-ITS reference database

To address these issues, a working group of the International Society for Human and Animal Mycology (ISHAM) on 'Barcoding of Medical Fungi' was established in 2011. The working group identified the necessity to: (i) generate a medical barcode database by incorporating existing fungal group-specific databases; (ii) extend the number of quality-controlled ITS sequences to cover all medically important fungal species; (iii) evaluate the value of ITS as a barcode at intra- and interspecies level, and (iv) eventually incorporate these sequences into the GenBank and other reference databases.

Fourteen mycology laboratories from three continents initially generated 3200 complete ITS sequences representing 524 clinically relevant species. The ISHAM-ITS reference database is freely accessible at http://its.mycologylab.org/ and http://www.isham.org/. It contains 226 species represented by one strain, 116 species by two strains, and 182 species by a minimum of three to a maximum of 115 sequences. The medically most relevant species are represented in the database by 20–115 strains. The lengths of complete ITS sequences in the ISHAM-ITS reference database range from 285 to 791 bp. The shortest complete ITS sequences are assigned to *Candida haemulonis* (285 bp), *Clavispora lusitaniae* (293 bp), and the longest ones to *Candida glabrata* (791 bp) and *Lichtheimia ramosa* (770 bp). The mean nucleotide length of ITS sequences in the database is 503 bp. The length, continuity and annotation of the ITS sequences have been checked using the software ITSx 1.0.7[7].

ITS intraspecies variation

The average intraspecies genetic diversity of the ITS region in medically relevant fungal species contained in the ISHAM-ITS database ranges between 0 and 2.25%, but in 170 species it is less than 1.5%. In 138 species it is less than 0.5%, in 27 species it ranges, between 0.5–1.0%, in five species (*Exophiala bergeri*, *Millerozyma farinosa, Histoplasma capsulatum, Candida pararugosa* and *Paracoccidioides brasiliensis*) between 1.01–1.5%, in four species (*Candida intermedia, Galactomyces candidus, Fusarium solani* and *Kodamaea ohmeri*) 1.5–2.0%, and in two species (*Lichtheimia ramosa* and *Clavispora lusitaniae*) it is more than 2% (Figure 1). The distribution of polymorphic sites revealed similar results. In 117 species, the number of polymorphic sites is less than five, in 35 species it is between five and ten, in 11 species between 11 and 15, in six species between 16 and 20 and finally more than 20 in seven species. The species with the highest number of segregating sites are *Cryptococcus albidus* (21 sites), the complex of *F. solani* (21 sites), *C. lusitaniae* (22 sites), *Candida glabrata* (22 sites), *K. ohmeri* (23 sites), *H. capsulatum* (38 sites) and *L. ramosa* (55 sites). Clinically important species have a low intraspecies variability in ITS regions making the ITS sequencing a useful genetic marker to be used for their identification. For the species with higher than 1.5% intraspecies diversity, additional molecular methods may be required for their reliable identification. Previous studies have shown that the genetic diversity of the ITS regions in fungi varies between taxa and that a universal cut off value to delineate species cannot be established[8]. Intraspecies diversity in medical fungi may be due to intra-genomic polymorphisms.

ITS interspecies variation

In 13 taxa, sharing the same phylogenetic clades, a clear barcoding gap (K2P[9] distance) was detected. This means that the highest intraspecies distances were smaller than the lowest genetic distances between species, generating a 'barcoding gap'. An example of taxa with and without barcoding gap is shown on Figure 2. The smallest barcoding gap (0.0002) exists in the *Microsporum* spp., while the largest one is present in the *Cladophialophora* spp. (0.09). However, four taxa have no clear barcoding gap: *Cryptococcus*,

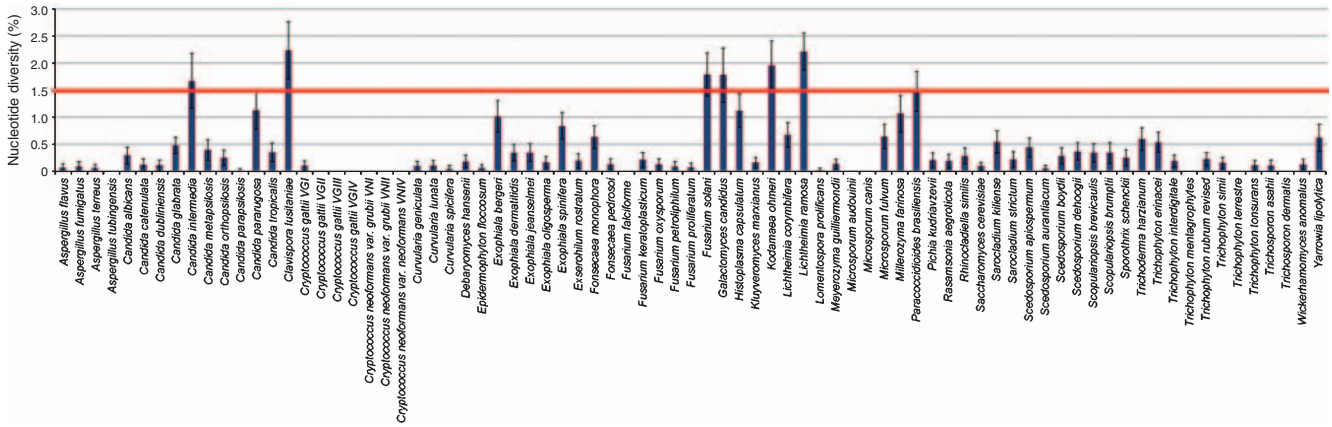

Figure 1. Average nucleotide diversity per species expressed as a percentage based on the value of π of the 79 clinically important fungal species. For *Cryptococcus neoformans* and *C. gattii* the variation is given for the major molecular types/potential species (VNI-VNIV and VGI-VGIV). The error bars indicate the standard deviation of nucleotide differences.

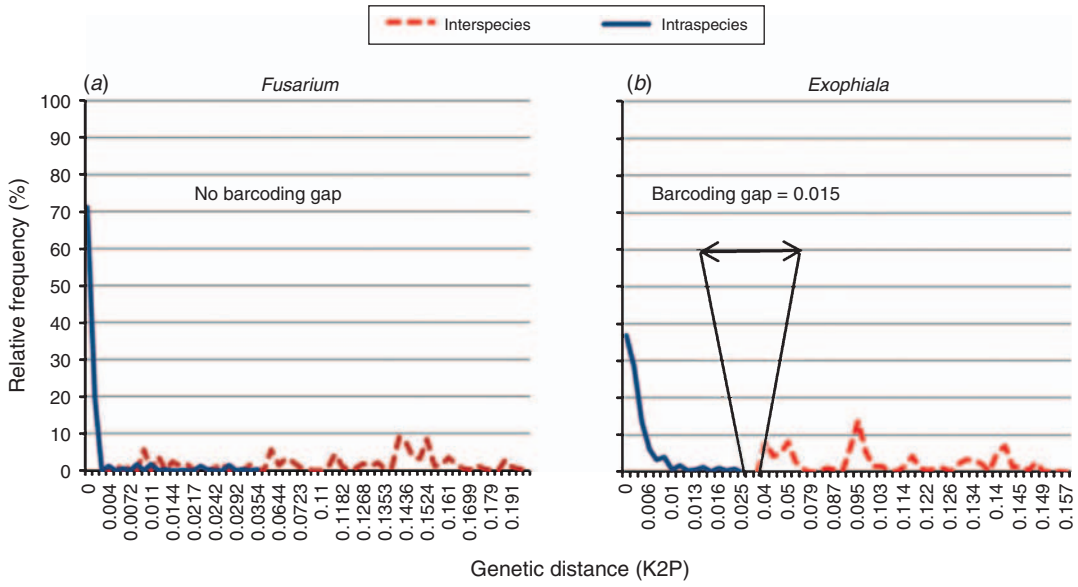

Figure 2. (*a*) Distribution of interspecies (red broken line) and intraspecies (blue solid line) pairwise Kimura 2-parameter genetic distances in *Fusarium* including *F. delphinoides; F. falciforme; F. oxysporum; F. proliferatum; F. solani; F. keratoplasticum; F. petroliphilum; F. verticillioides*. (*b*) Distribution of interspecies (red broken line) and intraspecies (blue solid line) pairwise Kimura 2-parameter genetic distances in *Exophiala* including *E. bergeri*; *E. dermatitidis*; *E. exophialae*; *E. jeanselmei*; *E. oligosperma*; *E. spinifera*; *E. xenobiotica*.

Fusarium, Scedosporium and *Trichophyton*. In these taxa, the correct identification to the species level may be problematic when only using the ITS region as a genetic marker. As such, additional genetic markers and/or molecular methods are required.

Linking the ISHAM-ITS database to GenBank and UNITE

As a result of the collaboration with NCBI, all sequences are submitted to GenBank where they are labelled specifically, indicating that they are part of the ISHAM-ITS database and that they are quality controlled sequences. The definition line of each ITS sequence submission in GenBank covers the current taxon name of the species, the original strain number and a unique 'ISHAM-ITS ID' identifier (e.g. MITS1; MITS2. . .) as follows: '***Acremonium acutatum* strain FMR 10368 isolate ISHAM-ITS_ID MITS1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence**'. Following a BLAST search in GenBank, the user can clearly identify the query sequence selecting the ISHAM-ITS record from the Blast hits list. In GenBank, each ISHAM-ITS record is directly linked to the ISHAM-ITS database where more metadata are available for the associated strain (Figure 3). Moreover, sequences selected from the ISHAM-ITS database expand the number of medically relevant species represented in the RefSeq Targeted Loci (RTL) ITS reference database at NCBI[10]. Of the 421 fungal species contained in the ISHAM-ITS database, 71 are represented by Type cultures and have been submitted to RTL at NCBI. Conversely, 281 RefSeq sequences representing Type and verified material have been added to the ISHAM-ITS database. The NCBI and ISHAM curators are working together to update the species names in response to ongoing taxonomy and nomenclatural changes. In addition to GenBank, the sequences are also submitted to the UNITE database[11], where they are specifically labelled and directly linked to the ISHAM-ITS reference database.

Value of the ITS as a fungal DNA barcode

Taking the current data into account, most of the medically relevant fungal species can be identified based on their ITS region, verifying its status as a primary standard DNA barcode for fungi. However, in some cases the ITS has limitations in differentiating species. There are two possible reasons for this: either the taxa are insufficiently studied or the ITS region is simply an inappropriate marker for discrimination between closely related species. To overcome these limitations alternative loci and/or molecular methods are required. The occurrence of taxa without a barcoding gap may also be explained by the fact that the algorithms used by the barcoding community to calculate the genetic distances (K2P) or the algorithm used in BLAST[12] for pairwise sequence matching between the query sequence and reference sequences represent different approaches from those commonly used for phylogenetic analyses.

The ISHAM-ITS database is intended to cover all clinically relevant fungal species. It is open for further sequence submission to expand coverage of medically relevant species with a sufficient number of strains, either via direct submission through the database or by

Figure 3. Example of a quality controlled *Cryptococcus gattii* ITS sequence record in the ISHAM-ITS database.

contacting the curators of the database at: laszlo.irinyi@sydney.edu.au or wieland.meyer@sydney.edu.au.

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The authors thank all contributors to the ISHAM-ITS database (http://its.mycologylab.org). This work was supported by a NHMRC grant #APP1031952 to WM.

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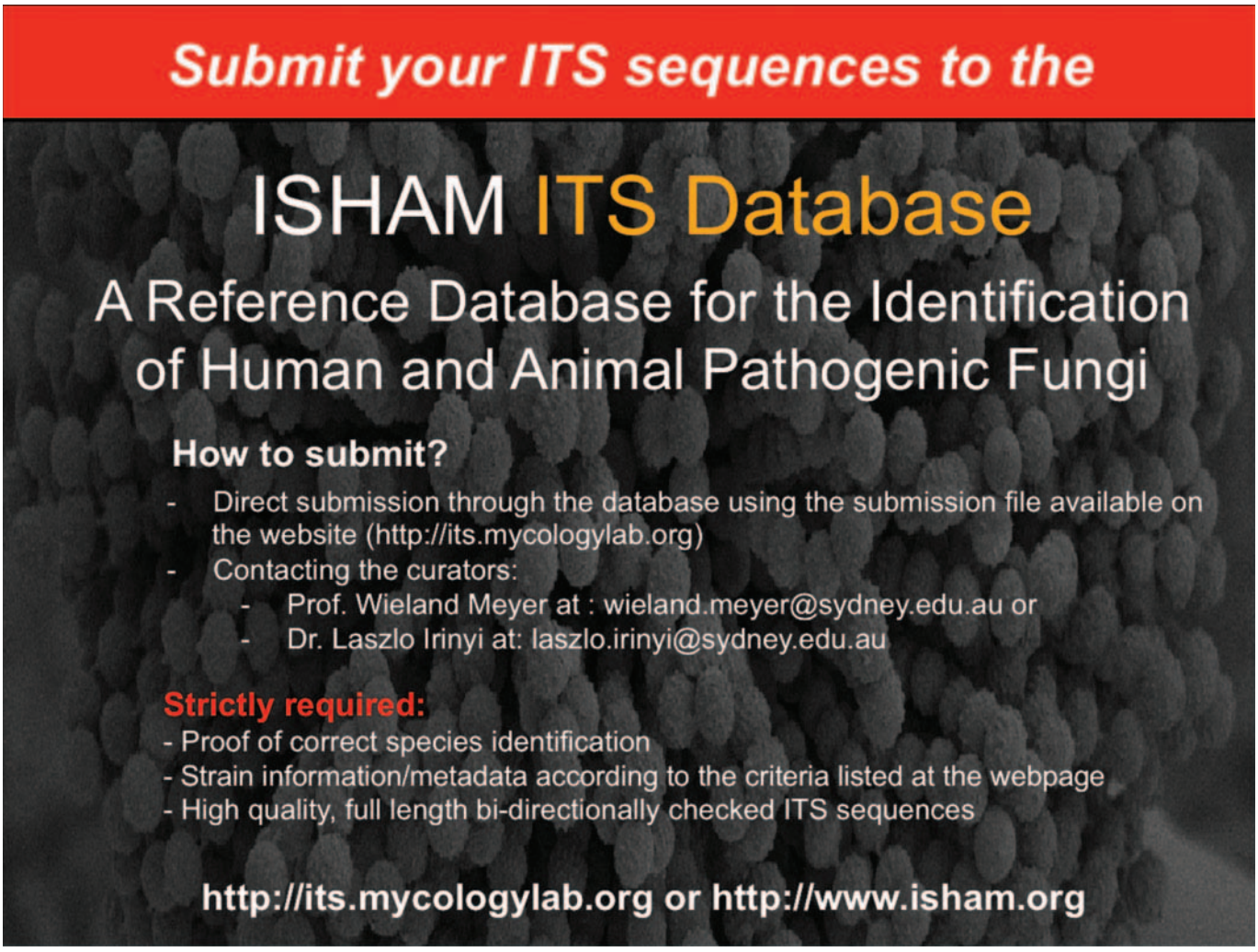
Biographies

Laszlo Irinyi is a Post-doctoral fellow in the Molecular Mycology Research Laboratory at the Center for Infectious Diseases and Microbiology, Westmead Millennium Institute. He completed his PhD on the phylogeny of *Didymellaceae* at University of Debrecen, Hungary. His research focuses on barcoding and molecular identification of human and animal pathogenic fungi. He is the curator of the International Society of Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database.

Professor Wieland Meyer is a Molecular Medical Mycologist and academic at the Sydney Medical School, The University of Sydney and the Fundação Oswaldo Cruz (FIOCRUZ) in Rio de Janeiro, Brazil, heading the Molecular Mycology Research Laboratory within the Centre for Infectious Diseases and Microbiology, Westmead Millennium Institute. His research focuses on phylogeny, molecular identification, population genetics, molecular epidemiology and virulence mechanisms of human and animal pathogenic fungi. He is the Convener of the Mycology Interest Group of ASM, the Vice-President of the International Society of Human and Animal Mycology (ISHAM) and a member of the Executive Committee of the International Mycological Association (IMA).



Phosphate theft: a path to fungal pathogenic success

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Inorganic phosphate/PO_4^{3-}/P_i is an essential and major constituent of numerous cellular components in all eukaryotes, including fungi. These components include nucleic acids, phospholipids and ATP. Despite its abundance in organic compounds, P_i is relatively scarce in its free form. To become successful pathogens, fungi must therefore acquire free P_i from the host environment via enzyme-mediated hydrolysis of P_i-containing molecules and/or via more efficient use of their own P_i. Fungal adaptation to a P_i-limited environment is governed by the phosphate (PHO) system, a cellular pathway consisting of P_i transporters, P_i mobilising enzymes and regulatory elements, such as kinases and transcription factors that respond to P_i levels. This system is well studied in the model non-pathogenic yeast, *Saccharomyces cerevisiae*, but not in fungal pathogens. In this review we present what is known about the PHO system in the model fungal pathogen, *Cryptococcus neoformans*, including our identification and characterisation of a secreted acid phosphatase, Aph1, which serves as a valuable reporter for identifying the less well-conserved PHO elements, including transcription factors.

Cryptococcus neoformans has restricted access to phosphate (P_i) during host infection

Cryptococcus neoformans is a deadly fungal pathogen with a high rate of morbidity and mortality worldwide[1]. Its pathogenicity is attributable to the production of a multitude of virulence factors, including a polysaccharide capsule, melanin and the enzymes phospholipase B/C and acid phosphatase (Aph1), which collectively promote host invasion, protection against host-derived stress, evasion of the host immune system and nutrient acquisition[2,3] (for review see Coelho *et al.*[4]). *C. neoformans* also provides a suitable model for understanding the PHO system in fungal pathogens. It has a fully sequenced genome that is highly amenable to manipulation and is currently being used by many laboratories around the world, including our own, to understand mechanisms of fungal virulence. *C. neoformans*, which infects the lungs and disseminates to the central nervous system via the blood stream and lymphatics, must obtain P_i from the host if it is to survive and become a successful pathogen, since mutant strains with reduced ability to either hydrolyse extracellular complexes containing P_i (acid phosphatase-deficient)[5] or transport free P_i across the plasma membrane[6] are hypovirulent in mice. Identifying the full repertoire of PHO responsive genes involved in the mobilisation of P_i from host and fungal sources, and their mode of regulation, is therefore of paramount importance. Knowledge of the PHO system in *S. cerevisiae* has led to the identification of some components of the PHO system in *C. neoformans*, including a secreted acid phosphatase Aph1, which is a useful reporter for identification of the less conserved elements, including transcription factors.

The PHO system in *S. cerevisiae*: a guide to understanding P_i homeostasis in fungal pathogens

The PHO system was extensively studied in *S. cerevisiae* and includes the high affinity membrane transporters (Pho84 and Pho89) system, polyphosphate (polyP) metabolising enzymes predominantly located in vacuoles, elements of the PHO regulation machinery and a number of PHO responsive genes involved in acquisition and storage of P_i. PHO-responsive genes include the high affinity P_i transporters and the secreted acid phosphatases,

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Pho5, Pho11 and Pho12, with Pho5 being the major secreted acid phosphatase. Excess cellular P_i is stored in vacuoles in chains of tens to hundreds of inorganic P_i molecules (polyPs), and is the first P_i storage source to be accessed during P_i deprivation. PolyP stores are believed to be exhausted prior to activation of the PHO system.

The *S. cerevisiae* PHO regulation machinery consists of the cyclin/cyclin-dependent kinase (CDK) complex Pho80/Pho85, the CDK inhibitor Pho81 and the transcription factor Pho4. During P_i deprivation, Pho81 represses the Pho80-Pho85 complex, preventing phosphorylation of Pho4. Hypophosphorylated Pho4 activates the expression of PHO responsive gene. Lee *et al* demonstrated that activation of the PHO system during P_i limitation paradoxically requires highly phosphorylated inositol species known as inositol pyrophosphates (PP-IPs)[7,8]. PP-IPs are comprised of inositol covalently linked to mono- and di-phosphate groups, and are derived from the mono-phosphorylated (IP) species. Exactly how PP-IPs are involved in PHO system regulation remains controversial. Lee *et al* established that the concentration of intracellular PP-IP_5 (IP_7) increased under P_i-deficient conditions and that IP_7 allosterically modulates the CDK inhibitor Pho81 leading to a conformational change in the Pho85-Pho80-Pho81 complex and reduced phosphorylation of Pho4[7,8]. However, Lonetti *et al.* demonstrated the opposite, with intracellular PP-IP levels decreasing by 80% during P_i deprivation[9]. Despite extensive investigation of the PHO pathway in *S. cerevisiae*, the mechanism for sensing intracellular P_i concentrations and the identity of the P_i sensor remain to be elucidated.

The PHO system in *C. neoformans* and its role in virulence

Only a few components of the PHO system have been identified in *C. neoformans* on the basis of their homology to PHO components from *S. cerevisiae* and responsiveness to the intracellular P_i status. These include the high-affinity P_i transporters (Pho840, Pho84 and Pho89)[6], a family of secreted and intracellular acid phosphatases (Aph1, Aph3 and Aph4) (Lev *et al*.[5] and our unpublished observation) that hydrolyse complex organic P_i sources, and the polyP polymerase Vtc4, which synthesises vacuolar polyPs[6].

***P_i transporters*:** A cryptococcal mutant defective in P_i uptake, *Δpho840Δpho84Δpho89*, is significantly attenuated for virulence in a mouse model of cryptococcosis, confirming that *C. neoformans* must acquire P_i from the host environment to reach its full virulence potential[6].

***Extracellular acid phosphatase Aph1*:** Extracellular P_i is often complexed to organic molecules and must be released by secreted acid phosphatases. The liberated P_i is then taken up by the P_i transporters. Acid phosphatase activity has been detected in the secretions of a large majority of *C. neoformans* strains isolated from patients with AIDS including the clinical type strain H99[10]. In a proteomic analysis of the H99 secretome, we identified the classically secreted acid phosphatase, Aph1, and deleted its encoding gene, creating *Δaph1*[5]. Using a chromogenic enzyme assay which measures hydrolysis of the synthetic substrate, para-nitrophenol phosphate (pNPP), we found that the *Δaph1* mutant was deficient in secreted acid phosphatase activity during P_i deprivation, confirming that Aph1 is the major secreted acid phosphatase in *C. neoformans*[5]. qPCR revealed that, similar to Pho5 from *S. cerevisiae*, Aph1 production during P_i deprivation is regulated at the transcriptional level. We also found that the concentration of P_i in a standard cell culture medium is sufficiently low to induce *APH1* expression providing further evidence that *C. neoformans* encounters a low P_i environment during host infection. *Δaph1* was less virulent in *Galleria mellonella* and mice, consistent with P_i mobilisation from complex sources being essential for virulence[5]. However, Aph1 deficiency had less of an impact on virulence than loss of the high affinity P_i transport system, suggesting that Aph1 hydrolyses only a proportion of the extracellular P_i sources available within the host. Other enzymes that potentially work in conjunction with Aph1 to achieve comprehensive mobilisation of P_i from complex sources include alkaline phosphatases, phosphodiesterases and serine/threonine/tyrosine phosphatases.

***A role of intracellular Aph1 in P_i mobilisation*:** We found that Aph1 hydrolyses a broad range of substrates, including glucose-1-phosphate, β-glycerol phosphate, adenosine monophosphate (AMP) and mannose-6-phosphate and prefers the aromatic amino acid phosphotyrosine to phosphoserine/phosphothreonine[5]. By tagging Aph1 with the red fluorescent protein DsRed we observed its transport to vacuoles and the cell periphery via endosome-like structures (Figure 1*A*)[5]. Aph1-containing endosomes were highly mobile and were often observed transiently contacting the plasma membrane and vacuoles, reminiscent of the kiss-and-run mechanism observed for synaptic vesicle release[11] (Figure 1*B*). Acid phosphatases are often found inside acidic vacuoles, an environment conducive to the working pH of Aph1 (pH 5). The dual targeting of Aph1 to vacuoles and the extracellular environment, and the broad substrate specificity of Aph1 is consistent with a role for Aph1 in releasing P_i from a wide range of both extra- and intracellular sources.

***Vacuolar PolyP polymerases*:** Interestingly, the recruitment of P_i from polyPs is dispensable for fungal virulence since a *VTC4* deletion mutant was as virulent as WT in a mouse infection model[6].

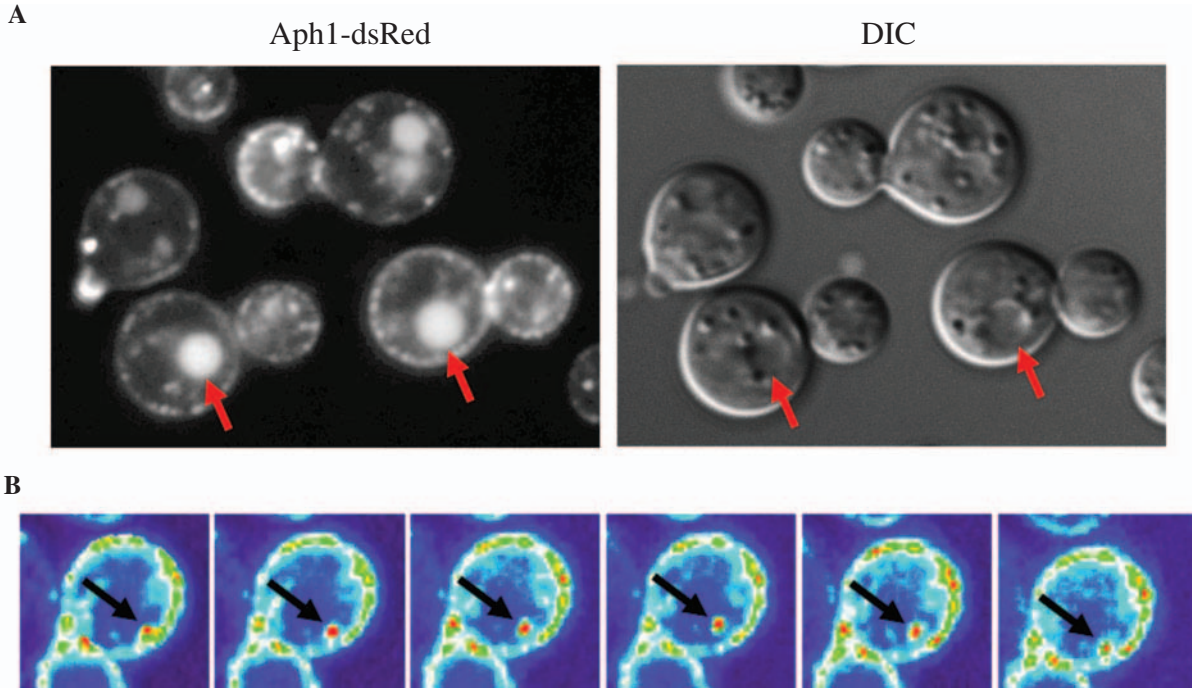

Figure 1. Acid phosphatase (Aph1) is localised to the cell periphery, vacuoles and mobile endosome-like organelles in *C. neoformans*. (*a*) Fungal cells expressing Aph1-dsRed fusion protein were starved of P_i for 3 hours and visualised with a deconvolution microscope. Vacuolar Aph1 is indicated by red arrows. DIC, differential interference contrast. (*b*) An endosome-like organelle containing Aph1-dsRed (black arrows) was followed using time lapse photography. The organelle is first observed contacting the membrane, retracting from it and then re-making contact in a 'kiss-and-run' type movement. Membrane contact potentially allows release of Aph1 into the periplasmic space. The images are presented using thermal scale to indicate fluorescence intensity, with red being the most intense.

***Intracellular acid phosphatases*:** *C. neoformans* also produces three intracellular acid phosphatases (Aph2, Aph3 and Aph4). *APH3* and *APH4*, but not *APH2*, are induced by low P_i (our unpublished observation). The decoupling of Aph2 from P_i regulation was unexpected given that Aph2 is most similar to Aph1. To test for potential up-regulation of other members of the APH family as a compensation for the loss of *APH1*, we measured *APH 2*, *3* and *4* mRNA in *Δaph1*, but found similar levels to WT (our unpublished observation), consistent with a lack of compensation. The contribution of each intracellular APH to cryptococcal virulence will be investigated by constructing single and combination deletion mutants.

***Pho transcription regulatory machinery*:** Probable PHO regulatory components with similarity to the cyclin-dependent kinase Pho85, the cyclin Pho80 and the cyclin-dependent kinase inhibitor Pho81 have been identified in *C. neoformans:* (CNAG_07871), (CNAG_01922) and (CNAG_02541) respectively[6]. However their role in regulation of the PHO system remains to be determined. Interestingly, no Pho4 transcription factor homologue has been identified in *C. neoformans.* The Bahn laboratory (Yonsei University, Korea) has created a cryptococcal transcription factor knockout library. Use of Aph1 as a reporter for screening this library should determine the identity of transcription factor(s) regulating PHO gene expression in *C. neoformans*.

***PP-IPs*:** Cryptococcal mutants deficient in IP_7 production also fail to induce *APH1* expression and secrete Aph1 during P_i deprivation (our unpublished observation). Via gene deletion analysis, we recently characterised the entire inositol polyphosphate biosynthesis pathway in *C. neoformans*, including the kinase directly responsible for IP_7 synthesis (Kcs1). Using the full set of kinase deletion mutants we also observed that IP_7 is essential for fungal virulence and dissemination to the brain in a mouse model (Lev *et al*.[12] and our unpublished observation).

In summary, little is known about how P_i homeostasis is regulated in pathogenic fungi, as compared with *S. cerevisiae*. We have identified and extensively characterised the major secreted acid phosphatase in *C. neoformans*, Aph1, which will provide a valuable reporter for identifying PHO system regulators in this important fungal pathogen. As individual components of the PHO system have been demonstrated to play a role in virulence, the investigation of P_i homeostasis in *C. neoformans* may also provide unique opportunities for antifungal drug development.

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Biographies

Dr Julie Djordjevic heads the Fungal Pathogenesis Group within the Centre for Infectious Diseases and Microbiology at the Westmead Millennium Institute, a research centre affiliated with the University of Sydney and Westmead Hospital. Using *Cryptococcus neoformans* as a model her research focuses on elucidating mechanisms used by fungi to cause systemic disease: specifically, understanding how fungal virulence factors get secreted and investigating the role of a series of newly described inositol polyphosphate kinases in a cellular pathway critical for production of virulence factors and phosphate homeostasis.

Dr Sophie Lev studied for her PhD degree in the Technion – Israel Institute of Technology, researching signal transduction in a fungal pathogen of corn. After completion of her PhD in 2003, she proceeded with post-doctoral training in the same University, and then in the University of California, Berkeley. She joined the Centre for Infectious Diseases and Microbiology in 2010, to work with Dr Julie Djordjevic and Professor Tania Sorrell to study signal transduction and virulence mechanisms in the medically important fungal pathogen, *Cryptococcus neoformans*.

Candida and macrophages: a deadly affair



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The human fungal pathogen *Candida albicans* is a significant cause of invasive disease in hospital patients. Treatments are inadequate resulting in high financial costs and mortality rates that approach 50%[1–5]. Over the past decades, extensive use of immunosuppressive therapies and invasive medical procedures has exacerbated the problem[6]. Recent advances have shed light on the intimate relationship between *Candida* and innate immune cells, which triggers rapid fatal infections[7–10]. In this review we focus on the dynamic interaction between *C. albicans* and macrophages, which act as front line defense against invading pathogens, and discuss a newly discovered deadly affair.

Developmental transitions allow *C. albicans* to adapt and survive in host niches

The capacity to undergo a reversible switch between a yeast and hyphal mode of growth is linked to the virulence of *C. albicans*[11,12]. Although other yeast species, such as the model yeast *Saccharomyces cerevisiae*, are capable of transitioning between different cell types, unlike *S. cerevisiae*, *C. albicans* not only grows as yeast and pseudohyphae, but it also makes true hyphae – highly elongated tubular cells with no constrictions between mother and daughter cells and a primary septum that is not degraded during cell division[13]. Hyphae enable *C. albicans* to conquer new environments, and mediate pathogenesis-related functions such as invasion of epithelial tissue during colonisation of mucosal surfaces and the movement of *C. albicans* from the gastrointestinal tract to the bloodstream[7,13].

Regulated gene expression is at the core of cellular pathways enabling the yeast-to-hyphae morphogenetic switch, and it is thought that the interchange between these cell types is critical for pathogenesis[13–16]. Although yeast cells are considered better suited for transport via the blood in disseminated disease, whereas hyphal cells have invasive capacity, there are still a lot of questions about the specific functions of these developmental states and how they are triggered. Hyphae can switch back to yeast which is seen as yeast cells budding from the hyphal filaments[17]. Genetic mutants that cannot revert to growth as budding yeast from the hyphal forms are attenuated in virulence[17], suggesting that the reverse transition is also important for pathogenicity.

In the human body, *Candida* must adapt readily to new environments as it transitions between host niches. It does so by orchestrating gene expression programs that control stress resistance, metabolic adaptation and morphogenesis[18,19]. The innate immune response is the primary and immediate response against candidiasis, and one leukocyte in particular, the macrophage, plays an important role[7,20]. The ability of *Candida* to switch between morphogenetic types is crucial for evasion of innate immunity[7,14,20]. When yeast forms of *C. albicans* are engulfed by macrophages, they can evade this line of defense by switching to hyphal growth, which leads to the hyphal filament bursting out of the macrophage and killing the host cell in the process[7,14,20]. Although it is tempting (and dramatic) to speculate that these hyphae kill by exerting pressure on the macrophage membrane and physically breaking through, recent reports from our lab and others suggest that other mechanisms, related to regulation of

the host–pathogen 'synapse', mediate host cell death and pathogen escape from macrophages[9,10].

Live cell assay of *Candida*-induced death of macrophages

Our lab has recently established powerful live-cell imaging to monitor the interaction of *C. albicans* with host macrophages using primary bone-derived macrophages from mice[9]. Using this *ex vivo* assay we could observe functionally distinct events over time in the *C. albicans*-macrophage interaction. When yeast forms of *C. albicans* are added at the start of the assay, they are rapidly phagocytosed by macrophages. Shortly afterwards, a switch from yeast to hyphae inside macrophages triggers macrophage death and, concomitantly, fungal hyphae become extracellular. This initial phase of macrophage killing is followed by a second phase of host cell death (Figure 1). We and others have recently identified genetic mutations in the host and in the pathogen responsible for the first phase of macrophage killing, as outlined below. Our ultimate goal is to understand precisely which *Candida* and host molecules are important to induce macrophage death, when they act temporally as the infection progresses, and what their contribution is to pathology and disease outcomes.

Phase I: *C. albicans* manipulation of the host macrophage

The key result that showed that *C. albicans* does not simply break macrophages, but rather induces a more regulated mechanism of killing, came with the realisation that macrophages derived from mice deficient in a central host immune pathway – the caspase 1 inflammasome – were protected from killing by *C. albicans* in the first few hours post-phagocytosis[9,10]. This was despite normal formation of fungal hyphae, and therefore it argued that physical disruption of macrophages by fungal filaments is not the only mechanism of host cell death. Instead, it appears that the initial hyphal formation triggers the macrophage to commit suicide by a programmed cell death pathway called pyroptosis, which is enacted by caspase-1 and occurs before the filament has extended to the surface of the macrophage (Figure 1)[9,10]. In addition to caspase-1, the inflammasome subunits ASC and NLRP3 are also involved in *Candida*-induced pyroptosis[10]. Pyroptosis is lytic, leading to

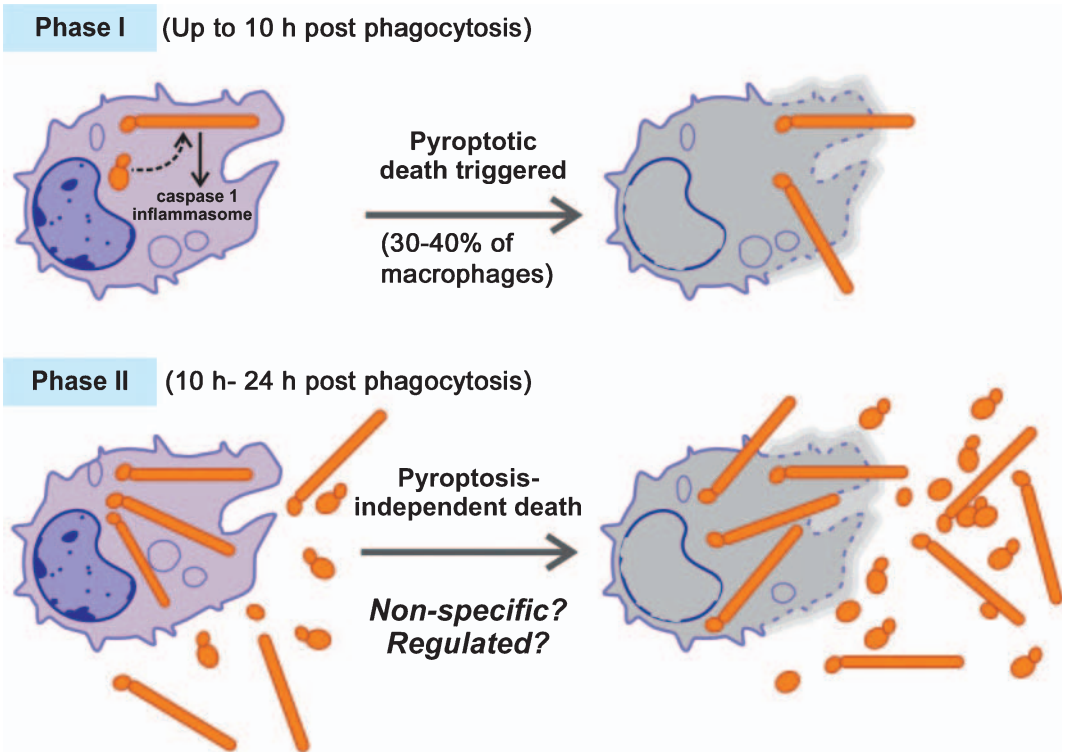

Figure 1. The two phases of *C. albicans* infection and killing of macrophages. Phase I. Initially after phagocytosis, *C. albicans* yeast cells transition to the hyphal form, which leads to activation of the caspase-1 inflammasome and pyroptotic death of macrophages. The lytic nature of pyroptosis leads to release of the *C. albicans* cells into the surrounding medium. The duration of the pyroptosis-dependent phase can be modulated by changing the multiplicity of infection (MOI). In the figure we present what occurs when the MOI of 6 *Candida* to 1 macrophage is used. Under these conditions, Phase I lasts for 8–10 hours post-phagocytosis. Only a fraction of macrophages are killed in Phase I (30–40%), and about half of the deaths are due to pyroptosis. Other potential mechanisms of *Candida*-induced macrophage cell death operating in Phase I remain to be understood. Phase II. As the interaction progresses, a second phase of host cell killing is initiated, leading to the death of the remainder of the macrophages commonly by 24 hours. In Phase II, escaped hyphae are seen surrounding the macrophages, and yeast-form cells appear as well. It is not presently clear whether Phase II is a regulated process, or a non-specific form of death that occurs due to large numbers of *C. albicans*.

bursting of the macrophage cell and release of *Candida*. We note that the macrophage-like cell line RAW264.7, which is commonly used to study *Candida*-macrophage interactions, does not express ASC[21], and therefore it is protected from pyroptosis following incubation with *C. albicans*[9]. While RAW264.7 cells, similar to caspase-1 deficient macrophages, are eventually killed by intracellular *Candida*[9], it is clear that an important component of *Candida*-macrophage interaction is not recapitulated in this model macrophage system. Pyroptosis is triggered as part of the inflammatory response to intracellular pathogens, to deplete the host niche needed for their replication and cause further immune activation[22,23]. Pyroptosis *per se* does not appear to be involved in the antifungal mechanisms exerted by macrophages, as *C. albicans* can survive in macrophages to the same extent in the presence or absence of pyroptosis[10]. As we have proposed, it appears that some pathogens, like *C. albicans*, can take advantage of this host response to evade being destroyed by macrophages[9,24]. In light of our studies, we suggest that the role of pyroptosis in *Candida* infections may need to be revisited, as caspase-1 triggers potent inflammatory signals that activate anti-fungal immune responses, but the same events are also associated with the pathology in fungal infections.

By using live-cell imaging we have observed that *Candida* kills macrophages in two phases (I and II) that are mechanistically quite different (Figure 1)[9]. The duration of the caspase-1-dependent Phase I of macrophage death is dependent on the infectious load of *C. albicans*: the bigger the ratio of *Candida* to macrophages, the faster transition to Phase II occurs (Wellington *et al.*[10] and our unpublished data). In our experiments, where six *Candida* cells per one macrophage were co-incubated, Phase I lasted for about 8–10 hours[9]. While this phase causes significant macrophage death, it is not complete. Approximately 30–40% of macrophages are killed, and these dead cells display subsequent nucleation of long hyphal projections outward as the imaging continues. Therefore, triggering of macrophage death is not uniform across all cells[9]. Inactivation of pyroptosis reduces macrophage death in Phase I, but does not fully protect[9,10], showing that *C. albicans* uses multiple means to escape from macrophages in the first phase of the interaction.

Key questions remain about how exactly *C. albicans* filaments are recognised by the caspase-1 inflammasome to trigger pyroptosis. An intriguing *C. albicans* genetic mutation identified in our lab is in the Srb9 subunit of the Mediator complex (a central eukaryotic transcriptional regulator). While this mutant strain is able to transition to long hyphal filaments in the macrophage during Phase I, these filaments cannot fully exert their function in causing macrophage death[9]. We have shown that Mediator is required for proper structuring of the cell wall[9,25]. The hyphae made by the *srb9* mutant displayed lower levels of exposed β-1,3 glucan on their cell surface, which is the main component of the fungal cell wall and is immunogenic[7,26]. The *srb9* mutant hyphae also showed altered biophysical properties[9], but how these cell surface changes are responsible for less macrophage cell death remains to be determined. Similarly, the transcriptional activator Upc2 is required for the ability of hyphal filaments to trigger pyroptosis[10]. Understanding the structural and physical properties of hyphae that are needed for signaling to the host macrophage to activate pyroptosis is the subject of current work. These factors may be reprogrammed by transcriptional regulators like the Mediator complex, as the developmental transition to hyphae begins inside the macrophage.

Phase II: macrophage necrosis or a regulated response?

Following Phase I, there is a second, quite rapid phase of macrophage killing that results in the death of the majority of remaining macrophages[9]. Very little is known about the mechanism of this second phase of killing. The second phase of killing can occur in the absence of caspase 1 or the alternative pyroptotic regulator, caspase 11[9,10]. This excludes pyroptosis as a mechanism of macrophage death in Phase II. It has been proposed that this second phase occurs when *C. albicans* numbers increase, as a non-specific process[10]. It is also possible that, like Phase I, Phase II is a regulated mechanism executed by a programmed host cell death pathway[9,24]. On the pathogen's side, it appears that hyphal filaments are functionally involved not only in the first phase, but also in the second phase of macrophage death. The Mediator mutant *med31*, which is delayed in making hyphae in macrophages, triggers significantly slower Phase II of macrophage death[9]. When assayed in *caspase 1/caspase 11* mutant macrophages, the *srb9* mutant of *C. albicans* also induced a slower rate of macrophage killing in Phase II, although much faster than the hyphae-defective *med31*[9]. Studying genetic mutations that uncouple hyphal morphogenesis from the ability to kill macrophages is likely to lead to better understanding of how *C. albicans* induces Phase II of macrophage death.

Concluding remarks

Candida albicans is a commensal organism of the skin and intestinal mucosa in approximately 50% of individuals. Breakdown of the physical barrier, due to surgery, burns or long-term use of antibiotics that reduces the numbers of competing microorganisms can lead to invasive *Candida* infections, even in immuno-competent patients. There is increasing evidence that systemic *Candida* infections trigger immunopathological reactions that contribute to the high mortality rate despite the use of state of the art antifungal therapy.

Dissecting the fungal factors that foster the transitions between yeast and hyphal forms, and host factors that recognise these forms, will undoubtedly reveal novel insights into the host–fungal pathogen synapse. By following these interactions on the molecular and cellular level, we will come to understand how our relationship with *Candida* can suddenly turn deadly.

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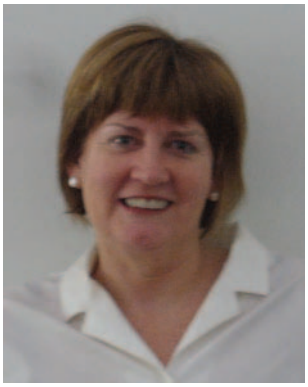
Biographies

Tim Tucey is a Research Fellow at Monash University. He obtained his PhD from the University of California, San Diego, where he studied telomerase in the budding yeast *Saccharomyces cerevisiae*. He is now applying his molecular and cell biology background to understanding the live cell dynamics of the pathogenic yeast *Candida albicans*.

Thomas Naderer leads the Macrophage-Pathogen Interactions laboratory in the Department of Biochemistry and Molecular Biology at Monash University. The Naderer lab focuses on understanding how microbial pathogens modulate host responses to egress from host cells and their contribution to disease.

Ana Traven heads the Laboratory for fungal pathogenesis in the Department of Biochemistry and Molecular Biology at Monash University. The research programs in the lab aim to decipher how *Candida albicans*, a common human pathogen, adapts to its environment and resists antifungal and host-derived attacks through metabolic control and by remodeling gene regulatory networks.

Clinical and Translational Mycology on the southern shores: perspective from the Australia and New Zealand Mycoses Interest Group



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The hosting of the 19th International Society of Human and Animal Mycology (ISHAM) Congress, the premier international forum for medical, veterinary and basic science mycology, in Melbourne, Australia in 2015, has prompted the opportunity to journey through the beginnings and rationale of coordinated and systematic study of clinical and applied mycology in Australia. The Australia and New Zealand Mycoses Interest Group (ANZMIG) is a special interest group of the Australasian Society for Infectious Diseases (ASID). This year, it has the honour of hosting, and the scientific organisation of, the 19th ISHAM Congress, which includes symposia sessions co-badged with the Mycoses Study Group, USA and the International Immunocompromised Host Society. Australian speakers make a strong contribution to the program in Clinical, Translational, Basic Science and One Health streams.

History and beginnings

The driving force behind the first ANZMIG meeting in 1997 was Emeritus Professor David H Ellis, then, Head of Mycology at the Women's and Children's Hospital, Adelaide and Ms Jennifer Antonino, area manager for Australia and New Zealand, Nexstar Pharmaceuticals who provided support and the first iteration of ANZMIG, the Mycoses Interest Group (MIG), was born. Starting with 8 members (including the authors), interest in MIG steadily increased and its name changed to ANZMIG to reflect the contributions of New Zealand colleagues. The inspiration of Professor Ellis and founding support from Ms Antonino and Nexstar Pharmaceuticals, through all the initial teething problems, cannot be overstated.

Today, ANZMIG scientific and business meetings are held every quarter in a major city and membership has expanded to represent scientists and veterinarians active in mycology but the focus is still on clinical and epidemiological aspects of mycology. Meetings are supported in part by each of Gilead Sciences, Pfizer and MSD Australia, with Astellas also becoming a sponsor in 2015. Registrars in training are encouraged to attend the scientific sessions, and three are selected to present cases or discuss their work. By prior arrangement, their presentation and literature review can be accredited by the Infectious Diseases Specialist Advisory Committee, Royal Australasian College of Physicians (RACP) as a project. ANZMIG activities are headed by a chair, immediate past chair, scientific chair and secretary.

Objectives and professional standing

ANZMIG has two main objectives. It is a professional body to facilitate the conduct of national mycology surveillance and clinical trials, including antifungal drug trials and antifungal guideline development. Its other primary goal is to promote and improve education in all aspects of mycology including the delivery of such education outside of Australia in the Asia Pacific region. Laboratory capacity building and training of scientists is likewise actively promoted. A summary of both its educational and clinical/translational research activities is given below.

Education

ANZMIG has conducted regular education programs, firstly as wet laboratory workshops at the Women's and Children's Hospital, Adelaide, Westmead Hospital and Concord Hospital, in Sydney.

The biennial Mycology Masterclasses, beginning in 2003, are a focus for mycology in Australia. Comprising a mix of basic, translational and clinical mycology as well as a relaxed and convivial atmosphere conducive to debate and discussion, these classes appeal to trainees and laboratory scientists, and include participation from haematology, intensive care and organ transplantation specialists seeking to update their knowledge. There has always been a waiting list for attendance. They are now coordinated by the current Secretary of ANZMIG, Dr Sarah Kidd, Head, National Mycology Reference Centre, SA Pathology. The 2015 Masterclass will be held in conjunction with the 19th ISHAM. Mycology Masterclass members are also faculty for the Asia Pacific Mycology Masterclasses supported by Merck since 2012. Many fruitful collaborations and exchanges of ideas have resulted from the Asia Pacific Mycology Masterclass. Its members also act as faculty for the Gilead CARE program on improving education and management of fungal infections.

ANZMIG has organised a popular session at the annual scientific meeting of ASID for 15 years and awards an annual prize for the best mycology abstract at that meeting. Its members also make active contributions to Australian Society for Microbiology and Australian Society for Antimicrobials and ASID clinical trials network.

Mycology surveillance

A major legacy of ANZMIG has been the collaborative network established for studies which have defined the epidemiology of serious fungal infections in Australia. This began with The Australian Candidemia Study in 2000 to which almost all microbiology laboratories in Australia contributed, and which resulted in publications describing the general epidemiology[1]. Special groups such as paediatrics, cancer, solid organ transplantation, intensive care and uncommon species were also described; essential data for developing antifungal guidelines for invasive candidiasis was described. A rich repository of well-characterised bloodstream isolates was established, curated by Westmead hospital and SA Pathology and available for future research. Recently the new CLSI breakpoints have been examined against this data set[2]. A follow-up to this study is underway now and we will compare epidemiology, susceptibility and outcomes to the earlier data.

An important local fungus *Scedosporium* spp., was chosen for the second epidemiological study and application of molecular testing to isolates allowed identification and clinical characterisation of *Scedosporium aurantiacum*, a relatively new species for which there was little clinical information including that of treatment[3].

Moulds other than *Aspergillus* were the focus of a more recent study (ongoing), showing that Mucormycetes were the most common, closely followed by *Scedosporium* species and identifying the need for more rapid diagnostic tests and definitions targeted to patients without classical immunocompromise[4].

Cryptococcus gattii epidemiology, another fungus well known in Australia was also described[5]. Important prognostic factors were identified for the first time and treatment responses characterised[5].

Although not ANZMIG studies, two successful NHMRC grants entailing several million dollars evolved from the ANZMIG collaborations: one a randomised trial of *Aspergillus* PCR and galactomannan (GM) as early diagnosis for aspergillosis in high risk haematology patients compared to standard diagnostic methods[6]. This study showed the safety of this approach. A second grant related to risk prediction for candidemia in the ICU is ongoing. Australian and New Zealand mycology suffers the same underfunding and low profile described internationally[7] and the ANZMIG group is working to improve NHMRC grant outcome funding.

Antifungal guideline development

ANZMIG members led the steering and writing groups for Australian and New Zealand Consensus guidelines for antifungal agents in the haematology/oncology setting. These guidelines were first published in 2004 as a standalone paper and updated in 2008 as a supplement consisting of six separate articles[8]. When recently compared with other international antifungal treatment guidelines, the 2008 guidelines ranked the highest overall when the Appraisal of Guidelines Research and Evaluation (AGREE) criteria for assessing the quality and methodological rigour of guidelines was applied[9]. A recent survey of antifungal drug prescribers also highlighted the clinical relevance and applicability of the previous guidelines[10]. These guidelines were updated most recently in 2014 and linked to Therapeutic guidelines Australia, the standard national hospital-wide antimicrobial prescribing guide.

The 2014 guidelines are the most comprehensive yet, incorporating nine sections and including recommendations for paediatrics, *Pneumocystis jiroveci*, *Cryptococcus gattii* as well as a survey of current diagnostic and prophylaxis practices[10–17]. Members of ANZMIG have contributed to other guidelines such as the International Society for Host and Lung transplantation.

After a slow start, and under the valuable mentorship of David Ellis, ANZMIG has expanded and been a successful collaborative group promoting education and research and education in

Mycology within Australia and our region. Attendance at the scientific sessions is open to all and is encouraged. Over the years, ANZMIG has had the honour of visiting clinicians, hospital scientists and academics attend, always imparting valuable advice to both the scientific content and business end of the special interest group. A continued goal is to increase the profile of mycology and improve grant funding success in this relatively neglected area.

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Biographies

Professor Monica Slavin is Head, Department of Infectious Diseases, Peter MacCallum Cancer Centre in Melbourne and leads the Immunocompromised Host Infection Service at Royal Melbourne Hospital. Her major research interest is in infections in the immunocompromised, especially patients with cancer and undergoing transplantation, with a focus on prevention and early treatment of invasive fungal infection. She is current chair of the Australia and New Zealand Mycoses Interest Group, ASID.

Associate Professor Sharon Chen is a Medical Microbiologist and Infectious Diseases Physician at the Centre for Infectious Diseases and Microbiology (CIDM), Westmead Hospital, with research interests in medical mycology, new diagnostic tests in microbiology and laboratory automation. She is currently the Director of CIDM-Laboratory Services at the ICPMR, Pathology West, and past chair and scientific chair of the Australia and New Zealand Mycoses Interest Group, ASID.

The Westmead Medical Mycology Collection: basis for research and diagnosis of fungal diseases




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The Westmead Medical Mycology Collection is completing 20 years of existence. During this time there have been 10,073 strains deposited representing 437 species, which are currently maintained in the collection. Established originally under the curation of Professor Wieland Meyer at the Molecular Mycology Research Laboratory, in the Centre for Infectious Diseases and Microbiology at the Sydney Medical School – Westmead Hospital, The University of Sydney, it recently moved to the new Westmead Millennium Institute for Medical Research in Westmead, Australia. Its primary aim is to preserve Australian human and animal pathogenic fungal biodiversity while providing reference and clinical strains for the mycology community. The stored strains are identified phenotypically, biochemically and molecularly. They are stored either lyophilised, in glycerol at −80°C or as living culture at 14°C. The majority of the stored strains are the result of specific clinical, molecular epidemiological and basic science projects. As such, the pathogenic yeasts *Cryptococcus neoformans* and *C. gattii* account for 54% of the specimens deposited. To further characterise the maintained strains specific MultiLocus Sequence Typing schemes have been developed for *C. neoformans*, *C. gattii*, *Scedosporium apiospermum*, *S. aurantiacum*, *S. boydii* and *Pneumocystis jirovecii*, which are publically accessible at http://mlst.mycologylab.org. The collection also formed the basis for the development of the quality controlled ISHAM-ITS sequence database for human and animal pathogenic fungi accessible at http://its.mycologylab.org.

The Westmead Medical Mycology Collection (WM culture collection)

The storage of biological specimens is crucial for the preservation of microbial biodiversity and offering to the scientific community a wide range of data that can be used for diagnostic comparisons, as well as clinical and basic research. During the past 20 years, the WM Culture Collection has been focused on the culturing and storage of fungal strains, collecting 10,073 strains, representing 425 human and animal pathogenic fungal species, isolated from clinical, veterinary and environmental sources, from 52 countries (Figure 1). The collection maintains 134 type cultures and all reference strains for the major molecular types of the *C. neoformans/C. gattii* species complex[1]. The strains are characterised by applying traditional phenotypic, biochemical and advanced molecular techniques. The collection was originally established under the curation of Professor Wieland Meyer at the Molecular Mycology Research Laboratory (MMRL) in 1995 based on an existing strain collection started by Professor Tania Sorrell at the Centre for Infectious Diseases and Microbiology (CIDM) at Sydney Medical School–Westmead Hospital, The University of Sydney. With the move of CIDM and MMRL to the new Westmead Millennium Institute for Medical Research in June 2014 the collection found also a new permanent home. The collection maintains strains from a number of national

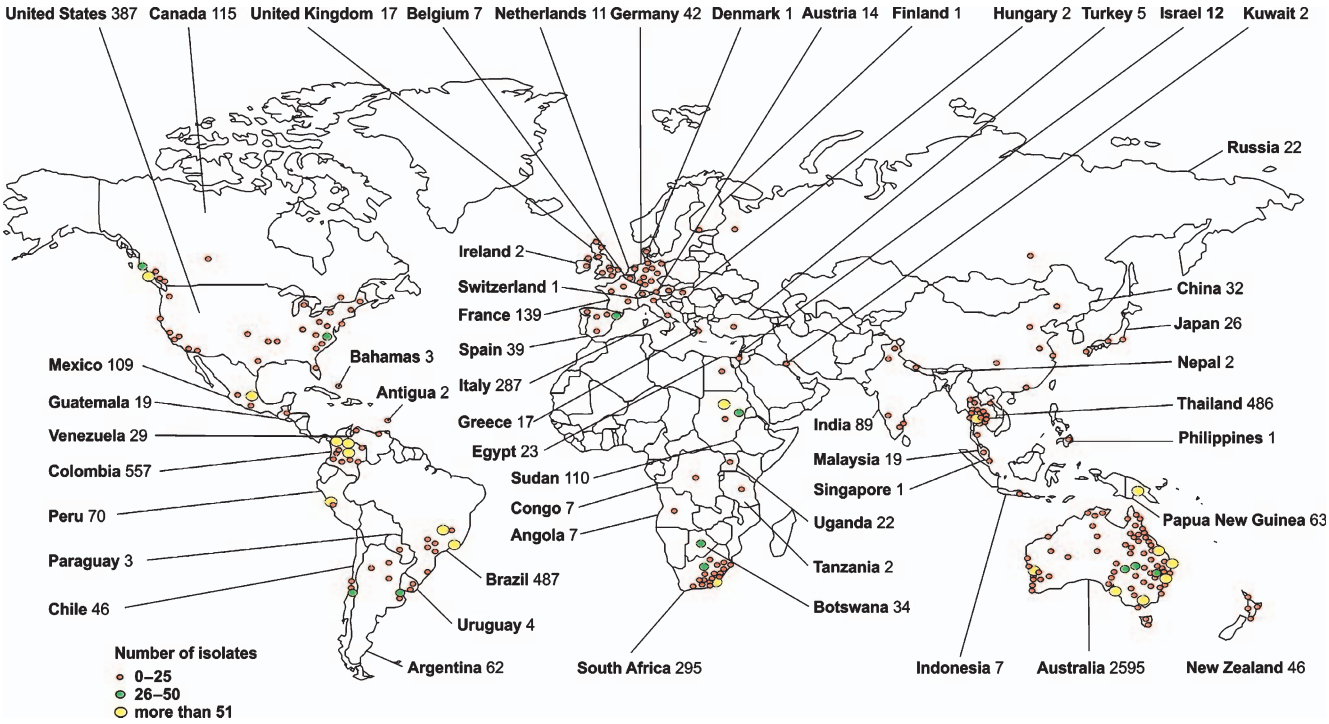

Figure 1. Origin of isolates present in The Westmead Medical Mycology Collection.

and international studies, including the Australian *Cryptococcus* studies, the Australian candidemia study, the Australian *Scedosporium* study and the Latin American and Brazilian cryptococcosis studies. The collection has close collaborations with other national culture collections at the SA Pathology, Adelaide, the Royal North Shore Hospital, Sydney, St Vincent Hospital, Sydney, Veterinary Pathology at the Faculty of Veterinary Sciences at Sydney University, Sydney and the PathWest – QEII Medical Centre, Perth and international collections in Austria, Argentina, Brazil, Chile, Colombia, France, Germany, Greece, Italy, Japan, Malaysia, Mexico, New Zealand, Peru, Portugal, Spain, South Africa, Thailand, Taiwan, The Netherlands and the USA. The collection is part of the Australian Microbial Resources Research Network (AMRRN), the Australian Microbial Resources Information Network (AMRiN) (http://amrin.ala.org.au/), the Council of Heads of Australian Collections of Microorganisms (CHACM), and of the Atlas of Living Australia (http://www.ala.org.au).

Strain storage

After samples are received, they are identified using phenotypic or biochemical methods and then a single yeast colony is selected to be subcultured on Sabouraud dextrose agar plates for 48 hours at 30°C and/or 37°C for DNA extraction and preparation for long-term storage. A loop of the strain is mixed with skimmed milk, inoculated in sterilised glass vials and then processed in the Alpha 1–4 LSC Freeze Dryer®. All samples are stored either freeze dried or at –80°C. Filamentous fungi are morphologically identified and then subcultured on Sabouraud dextrose agar for 48 hours at 20°C for DNA extraction and preparation for long-term storage. A 4 cm^2 section of the media is removed and inoculated in sterile glass vials containing 1 mL of sterile water, labelled and stored at 20°C. Metadata of the strains are stored electronically using the software package BioloMICS (www.bio-aware.com, Hannut, Belgium), that keeps track of the number of stock in the collection and manages new strains using the stock management system.

The collection and molecular epidemiological studies

The genus *Cryptococcus* accounts for the highest number of samples (54%), with *C. neoformans* and *C. gattii* representing 80.5% of those species (Figure 2). This reflects one of the major research themes of the MMRL to understand the molecular epidemiology and virulence profiles of the etiological agents of cryptococcosis, considered one of the most common invasive fungal diseases in humans and responsible for more than 1 million cases per year and around 650,000 deaths in sub-Saharan Africa[2]. Within the 5,465 cryptococcal isolates available in the WM culture collection, 618 strains are typed using the International Society for Human and Animal Mycology (ISHAM) MultiLocus Sequencing Typing (MLST) consensus scheme[1] (http://mlst.mycologylab.org) and whole genome sequencing was performed for 119 strains[3] (Meyer and Firacative, unpublished data). All *C. neoformans* major molecular types (VNI, VNII, VNB, and VNIV) and 56 out of 324 sequence types (ST) currently described are present in the WM culture collection. All *C. gattii* major molecular types (VGI, VGII, VGIII, and VGIV) and 118 from the 336 STs described for *C. gattii*

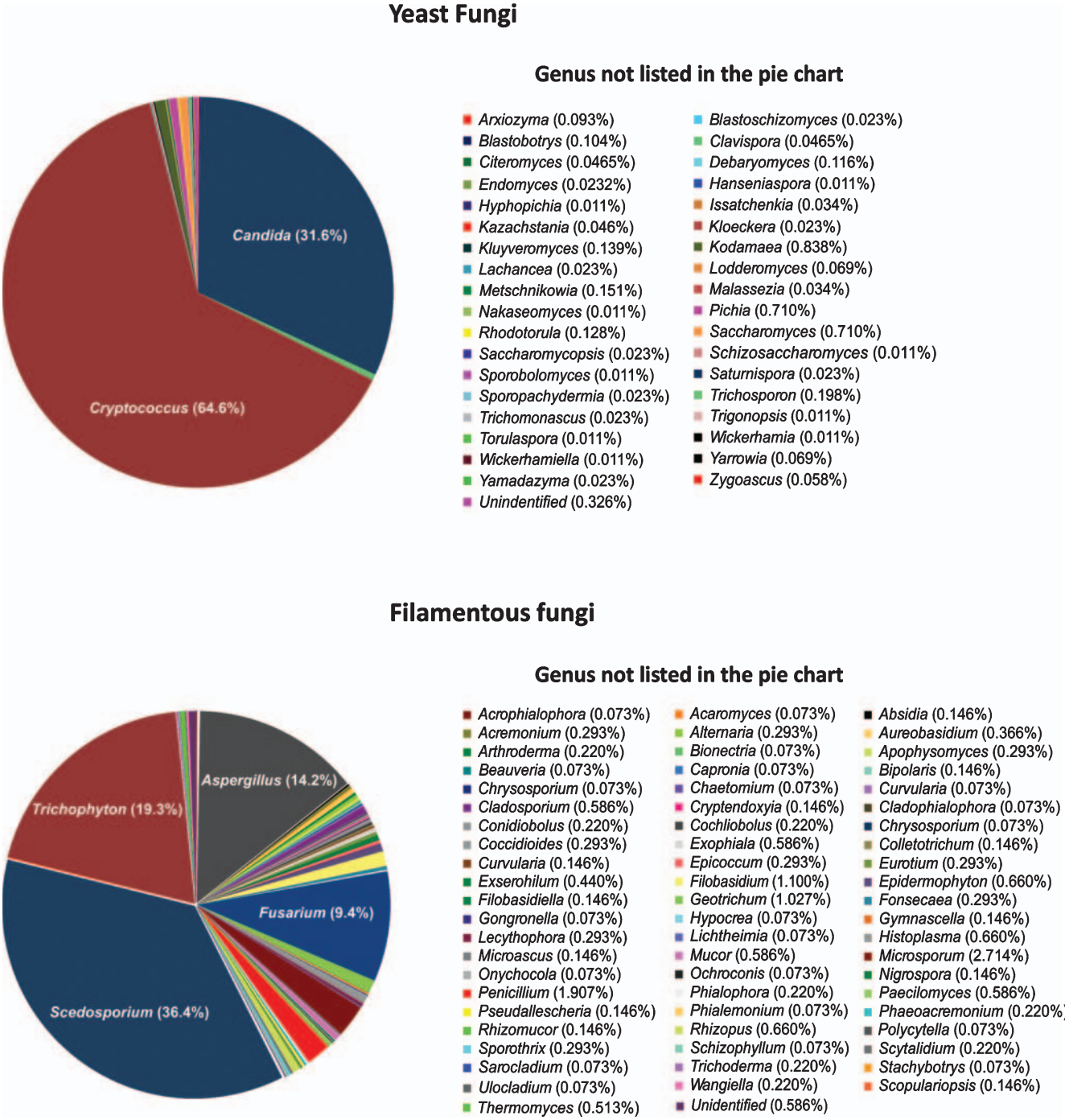

Figure 2. Distribution of Yeast and Filamentous fungi in The Westmead Medical Mycology Collection according to genus.

are available in the WM culture collection. *Candida* isolates represent the second most representative genus within the WM culture collection (122 species and 2,719 isolates), several of them were typed by molecular techniques. Filamentous and dimorphic fungi, especially those described as human pathogens including *Aspergillus*, *Fusarium*, *Penicilium, Pseudallescheria*, *Fonsecaea* and *Histoplasma*, are deposited as well. Among the filamentous fungi maintained in the collection the major pathogenic *Scedosporium* spp., opportunistic agents involved in pulmonary infections accounting for 497 strains (Figure 2), for which also specific MLST schemes, containing five genes[4] have been developed, and are available at http://mlst.mycologylab.org, to enable a global molecular epidemiology survey of *S. apiospermum*, *S. aurantiacum*, and *S. boydii*.

The collection and molecular identification of fungal species

Since fungal identification and taxonomy has remarkably improved during the last decade several recognised species such as *Fusarium solani, Paracoccidioides brasiliensis*, and *Sporothrix schenckii* have been distinguished as complexes of cryptic species. In this

context, the sequencing of the ribosomal regions, such as the Internal Transcribed Spacer (ITS), has been used for fungi identification for more than 10 years[5]. The ITS region was shown to be the most variable region within the ribosomal locus, being able to distinguish most closely related species, and as such has been selected as the universal fungal DNA barcode in 2012[6]. It has been used frequently for phylogenetic studies[5–7] and in the Assembling the Fungal Tree of Life (AFTOL) projects (http://tolweb.org). The WM culture collection formed the core unit of a global mycology research network combining 14 leading medical mycology laboratories, to establish the first quality controlled ITS database, the ISHAM-ITS reference database, which is available either via the ISHAM website at http://www.isham.org/ or directly at http://its.mycologylab.org. The database is constantly extended and accounts now for more than 3,000 sequences. More than 900 isolates from the WM culture collection are reference strains for the ISHAM-ITS database, 28.8% and 20.4% being *Cryptococcus* spp. and *Candida* spp., respectively. The remaining isolates represent the diversity of the fungal kingdom and etiological agents of mucormycosis, such as *Lichtheimia corymbifera* and invasive fungal infections, such as the rare *Blastobotrys proliferans*. The WM culture collection formed the basis for the development of a large number of molecular and MALDI-TOF based identification methods for human and animal pathogenic fungi, including: pan-fungal PCR[8], genus/species-specific PCR[9], real-time PCR[10], reverse line blots[11], rolling circle amplification[12] and MALDI-TOF[13].

The general mission of the WM culture collection is to continue to preserve and provide the mycology community with the Australian and global biodiversity of human and animal pathogenic fungi and associated metadata for clinical and basic research at a national and international level.

Acknowledgements

We thank all the members of the Australian and global mycology community that placed their trust in the WM culture collection and contributed to the construction of this important resource of biological diversity of human and animal pathogenic fungi.

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Biographies

The biography for **Professor Wieland Meyer** is on page 48.

Krystyna Maszewska is a research assistant at the MMRL who has graduated in Poland. She is managing the WM culture collection and carries out molecular identification using ITS1/2 and D1/D2 sequencing and genotyping of pathogenic fungi using PCR-fingerprinting, *URA*5-RFLP and MLST.

Aziza Khan is a research assistant at the MMRL who has completed her MSc in Medicine, with a focus on Infectious Diseases and Immunology at the University of Sydney. She performs ITS1/2 sequencing for the identification of pathogenic fungi for the development of the ISHAM-ITS database and conducts sequences for the selection of potential alternative DNA barcodes. She is working on the stability of *Cryptococcus* hybrid strains and conducts virulence studies for of various human pathogenic fungi using mice and *Galleria mellonella* larvae.

Kennio Ferreira-Paim is a Post-doctoral fellow in the MMRL at the CIDM, Westmead Millennium Institute and a Biomedical Scientist at the Clinical Hospital of the Triangulo Mineiro Federal University, in Uberaba, Brazil where he recently concluded his PhD in Tropical Medicine and Infectious Disease. His research focuses on the molecular epidemiology of *Cryptococcus* spp. and studying the molecular basis of fungal virulence using gene knockout and reconstitution and animal virulence models. He is a CAPES Science without borders visiting fellow (#9313133) from Brazil.

The role of clinical mycology reference laboratories



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Mycology reference laboratories fulfil a critical role in clinical microbiology. Staff with extensive training and expertise in mycology: (1) perform testing (e.g. fungal identification, susceptibility testing, fungal antigen detection, fungal DNA detection) using methodologies conforming to internationally accepted standards; (2) provide education and training in mycology; (3) provide scientific advice; (4) collaborate with regulatory bodies to deliver quality assurance programs; and (5) participate in research including monitoring emerging fungal trends and outbreaks. It is critically important that reference laboratories be resourced adequately for these functions and we believe there is scope to form an officially recognised network of mycology centres in Australia.

Importance of clinical mycology reference laboratories

The incidence and mortality of life-threatening fungal infection are on par with that of tuberculosis and malaria globally[1,2], and prompt, accurate diagnosis is critical to improve patient outcomes. However, variation in patient demographic and population size around the country, budgetary limitations, and a general decline in mycology expertise, means it is neither possible nor practical for many laboratories to maintain adequate staffing and resources to perform the full range of tests required for diagnosis of fungal disease. A reference laboratory fills this gap for less resourced laboratories at a state and/or national level. Less tangible, but just as important, reference laboratories provide leadership in their respective discipline and actively encourage other laboratories to improve their skills, knowledge and service delivery. This is largely achieved by providing sound clinical and scientific advice, education, and training materials. Such expertise includes interpretation of antifungal susceptibilities, updates in taxonomy and nomenclature, and developing and validating new diagnostic tests.

Defining mycology reference laboratories

After finding considerable variation in definitions and functions of reference laboratories for communicable diseases across member states of the European Union, the European Centres for Disease Control and Prevention (ECDC) identified and clarified their role, in a technical report designed for strategic planning, strengthening and maintaining a coordinated European reference laboratory network[3]. Based on this report, the core functions of mycology reference laboratories include provision of reference diagnostic services such as yeast and mould identification, antifungal susceptibility testing, serological and molecular detection of fungal pathogens; creating and maintaining resources such as fungal culture collections, books, and online tools; provision of scientific advice;

monitoring trends such as outbreaks, emerging pathogenic fungi and antifungal resistance; collaboration and research.

It is unclear how many mycology reference laboratories are actively operating in Australia due to a lack of formal recognition at the state or federal level. This is in contrast to the Australian Mycobacterium Reference Laboratory Network and the United Kingdom Clinical Mycology Network (UKCMN)[4]. The UKCMN has a steering committee and comprises a four-tiered classification of laboratory capabilities. At the base are local microbiology laboratories with the capacity to identify common yeasts and moulds; followed by specialist microbiology laboratories servicing tertiary hospitals with haematology, HIV and transplant services; to regional mycology centres which are essentially reference laboratories; capped off by a single laboratory, directly funded (in part) by Public Health England to provide a national service, investigate fungal outbreaks, curate a national collection of fungi, and set the standard for mycology laboratory quality assurance and training[4].

To our knowledge two Australian laboratories currently operate at a level that would be classified as a 'reference centre' by the UKCMN criteria: the National Mycology Reference Centre at SA Pathology, Adelaide, and the Clinical Mycology Reference Laboratory at Westmead Hospital, Sydney. A summary of the tests and functions of each of these laboratories is provided in Table 1. However, there are no laboratories in Australia that meet the UKCMN criteria for the highest level of mycology reference laboratory, mainly due to insufficient staffing by dedicated mycologists and a lack of direct funding.

Needs of clinical mycology reference laboratories

In order to perform its functions it is critical that mycology reference laboratories are recognised at the state and national level for their services, to have a mandate to perform them, be resourced accordingly, and are continuously assessed on their capabilities to perform these functions. Necessary resources include: (1) dedicated staff with extensive and ongoing training in laboratory mycology to an international standard; (2) appropriate infra-structure, including containment facilities, and equipment suitable for handling Risk Group 3 (RG-3) fungi; and (3) suitable materials, equipment and time to perform the functions of the laboratory. Critically, reference laboratories require sufficient funding to support and guarantee continuous and qualified work. The Australian mycology reference

Table 1. Summary of services and functions of the two currently active Australian mycology reference laboratories.

Service or function	NMRC	CMRL
Yeast identification and susceptibilities	✓	✓
Mould identification and susceptibilities	✓	✓
DNA sequence based identification	✓	✓
Aspergillus antigen detection (Galactomannan)	✓	✓
Fungal antigen detection (β-D Glucan)	Currently not available in Australia	
Histoplasma antigen detection	Currently not available in Australia	
Aspergillus PCR (EAPCRI compliant)		✓
Pneumocystis PCR	✓	✓
Panfungal PCR		✓
Dimorphic fungus precipitins		✓
Collaboration for quality assurance programs	✓	
Active research programs	✓	✓
Actively involved in education and training	✓	✓
Culture collection	✓	✓
Producing training materials (e.g. websites, laboratory guides)	✓	

NMRC, National Mycology Reference Centre, SA Pathology, Adelaide; CMRL, Clinical Mycology Reference Laboratory, Westmead Hospital, Sydney; EAPCRI, European *Aspergillus* PCR Initiative.

centres currently have to function within their budget for routine (local) diagnostic work. Since the cost of most specialised testing far exceeds the Medicare Benefits Schedule, invoicing referring laboratories for reference work is necessary but may prevent or discourage the appropriate utilisation of reference laboratories.

Best use of mycology reference laboratories

Both public and private laboratories may have personnel skilled in basic identification of yeasts and moulds, but may not have the resources for molecular identification of rare or poorly/non-sporulating moulds, or the facility to handle possible RG-3 pathogens. In these situations, the reference centres should be utilised. Antifungal susceptibility testing based on the 'gold standard' broth microdilution method (including commercially available Sensititre YeastOne) upon which clinical breakpoints are based, may be required in some situations rather than relying on the non-standardised minimum inhibitory concentrations and interpretations generated by Vitek, eTest or disk diffusion.

Pathology stewardship is important and reference laboratories should be consulted on appropriate selection of specimens and test requests prior to referral. An experienced medical mycologist and/or specialist pathologist overseeing laboratory activities is critical in this regard. This may include assessing the need for antifungal susceptibility testing (avoiding referrals of likely contaminants); use of *Aspergillus* PCR versus panfungal PCR, and the value of these tests on different specimen types such as bronchoalveolar lavage, blood, or tissues.

A good relationship and communication between reference laboratories and clinicians is essential for determining the most appropriate testing and in the interpretation of results. While there is co-operation and communication between the major mycology laboratories in Australia, this is not a formally recognised network, and there is limited representation of diagnostic mycology and reference laboratories at the government level. The Australia and New Zealand Mycoses Interest Group (ANZMIG) Laboratory Working Group is well placed to promote the value and resource requirements of mycology reference laboratories.

Acknowledgement

We thank Dr Elizabeth Johnson from the Public Health England Mycology Reference Laboratory, Bristol, UK, for providing information and advice.

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Biographies

Sarah Kidd is the Head of the National Mycology Reference Centre at SA Pathology, and a Lecturer at University of Adelaide and University of South Australia. She is Secretary of the Australia and New Zealand Mycoses Interest Group (ANZMIG) and Co-Chair of the ISHAM 2015 Congress Organising Committee.

Catriona Halliday is the Senior Scientist in charge of the Clinical Mycology Reference Laboratory at Westmead Hospital where she has been working for over 14 years. Her research interests have focused on the development and implementation of culture independent tests to aid in the rapid diagnosis of invasive fungal infections, in particular invasive aspergillosis.

David Ellis is an Emeritus Mycologist in the National Mycology Reference Centre at SA Pathology and an Associate Professor in the School of Molecular and Biomedical Science at the University of Adelaide.

Australia in the global picture of the molecular epidemiology of *Cryptococcus gattii* molecular type VGII





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Cryptococcosis, a life-threatening disease of the lung and central nervous system of humans and a broad range of other animals, is caused by the basidiomycetous yeasts *Cryptococcus neoformans* and *C. gattii*[1]. Although most cases of infection in the world are caused by *C. neoformans*, there is an important prevalence of *C. gattii* among clinical and veterinary samples in Australia[2–4] and the natural habitat of the yeast is strongly associated with native *Eucalyptus* species[4,5], which together position Australia as an endemic area for the less common cryptococcal species. From the numerous *C. gattii* infections that have been reported in Australia, the molecular type VGII, amongst the four recognised molecular types (VGI-VGIV), has been associated with a high occurrence and a number of small cryptococcosis outbreaks, with most of the isolates belonging to the clonal subtype VGIIb[2], which was initially described in 1999 causing part of the ongoing cryptococcosis outbreak on Vancouver Island, British Columbia, Canada[6]. These findings indicate that Australia is an important stepping-stone in the global dispersion of this outbreak-related subtype and highlight the need for continuous surveillance.

Since it was first reported in Vancouver Island and because of an increased number of human and veterinary *C. gattii* infections, previously uncommon in North America[6,7], *C. gattii* has been the focus of several studies conducted to elucidate the underlying evolutionary mechanisms involved in its emergence and dispersal in temperate climates and to determine the potential geographical origin of this fungus. At first, two distinct subtypes among the molecular type VGII isolates responsible for the Canadian outbreak were characterised by multilocus sequence typing (MLST), the clinical and environmental common highly virulent VGIIa subtype, and the rarer, less virulent VGIIb subtype[8]. The early finding that both subtypes have only mating type alpha isolates that were fertile, led to the suggestion that same-sex mating between two alpha cells was the driving force for the emergence of the outbreak[8]. Within a decade, an additional novel VGII subtype, VGIIc, also with increased virulence, but not associated with the Vancouver Island outbreak,

was identified in the Pacific Northwest, which emphasised the continuous emergence of new genotypes among *C. gattii*[7].

MLST analysis[9] of a larger number of globally collected clinical, veterinary and environmental isolates showed that the subtypes VGIIa and VGIIb are not only present in North America, but also in South America and Europe, with the subtype VGIIb especially being also found in Asia and Australia[2,10,11]. Supported by the early association reported between *C. gattii* and *Eucalyptus* species[4,5] and the extensive introduction of these trees to different places in the world, such as California in the USA, the identification of the subtype VGIIb in Australia, suggested an Australian origin of the outbreak isolates[8]. However, the presence of VGIIa and VGIIb in South America and the close association of *C. gattii* with a number of tropical trees that has been reported in this region, indicated strongly that both genotypes originated from South America and have since then been dispersed and introduced on several occasions to other parts of the world, including North America[10].

To shed light on the speculations that have arisen on the origins of the outbreak strains, the Molecular Mycology Research Laboratory at the University of Sydney is leading an ongoing research collaboration among different institutions in Australia, South and North America, Asia, Africa and Europe to carry out global epidemiological studies based on MLST typing[9]. When looking worldwide at the geographical distribution of the genetic diversity, shown by this approach, the highest number of sequence types has been detected so far in South America, while Australia harbors only few sequence types (Figure 1). High levels of genetic diversity are a strong indication of ancestral origins, hence, this extended genetic diversity seems likely to corroborate the idea that the Vancouver Island outbreak subtypes have originated from South America[2,10]. In contrast, the low genetic diversity combined with the over-representation of some sequence types in Asia and Australia suggest that a clonal expansion has occurred and that the colonisation of such clones may lead to the development of local outbreaks, such as the one observed in a group of sheep in Western Australia[4].

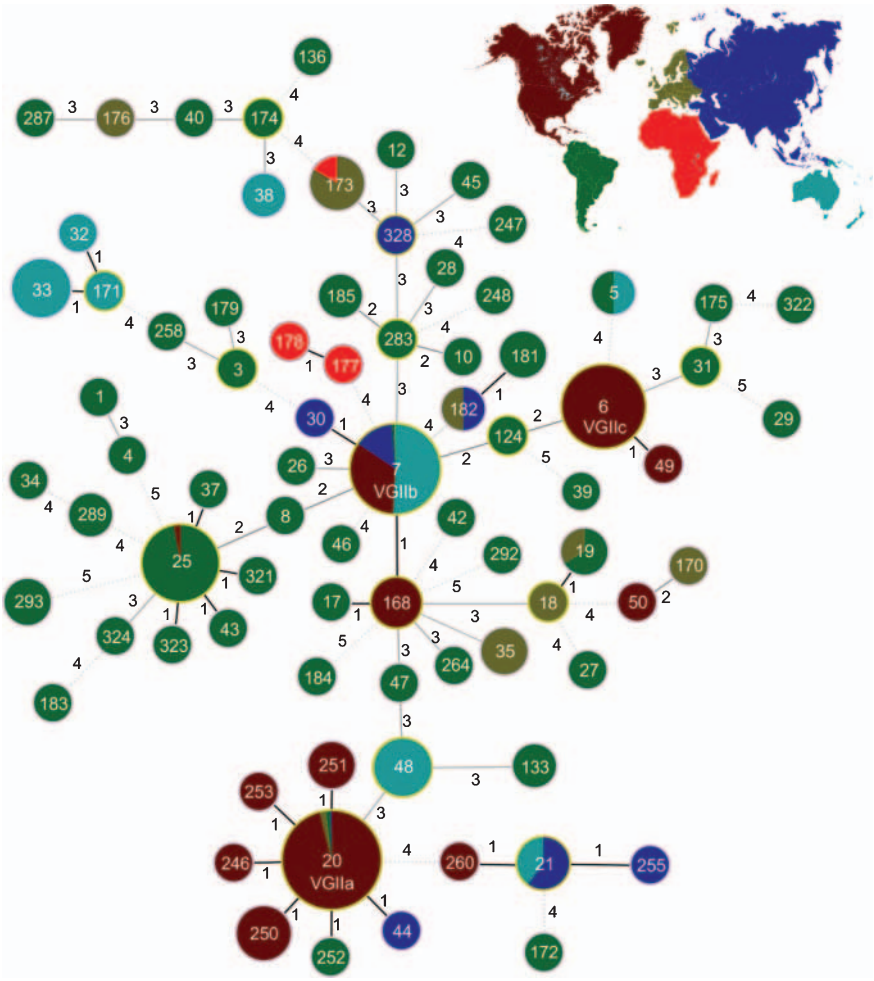

Figure 1. Minimum spanning tree of global *Cryptococcus gattii* VGII isolates representing the continent where the different sequence types (ST) have been identified. The tree was calculated by goeBURST analysis implement in Phyloviz software (http://www.phyloviz.net/wiki/) and which used the same priority rules for linking STs as eBURST but with a global optimisation. A total of 78 STs representing 529 isolates was included. Size of the circles corresponds to the number of isolates within each ST. Number of nucleotide polymorphisms are shown on each branch. Solid, grey and dashed branches represent at least one, two to three and more than 4 differences, respectively. The three subtypes VGIIa (ST20), VGIIb (ST7), and VGIIc (ST6) are highlighted in the picture while the remaining circles represents the other VGII lineages.

Taking advantage of the more comprehensive view obtained by investigating the whole genome, whole genome sequencing (WGS) was recently performed on 118 *C. gattii* VGII isolates from five continents, representing most of the MLST haplotypes previously identified[2,10–12]. Even though the subtypes from the Pacific Northwest of the USA were completely clonal (Figure 2), as shown already by MLST (Figure 1), but with the much greater resolution obtained by WGS, they showed various genetic differences with the other VGII lineages, including mutations, deletions, transpositions, recombination events, and gene content differences which are potentially related to habitat adaptation, virulence, and pathology[12]. Presence or absence of genes among the different isolates is

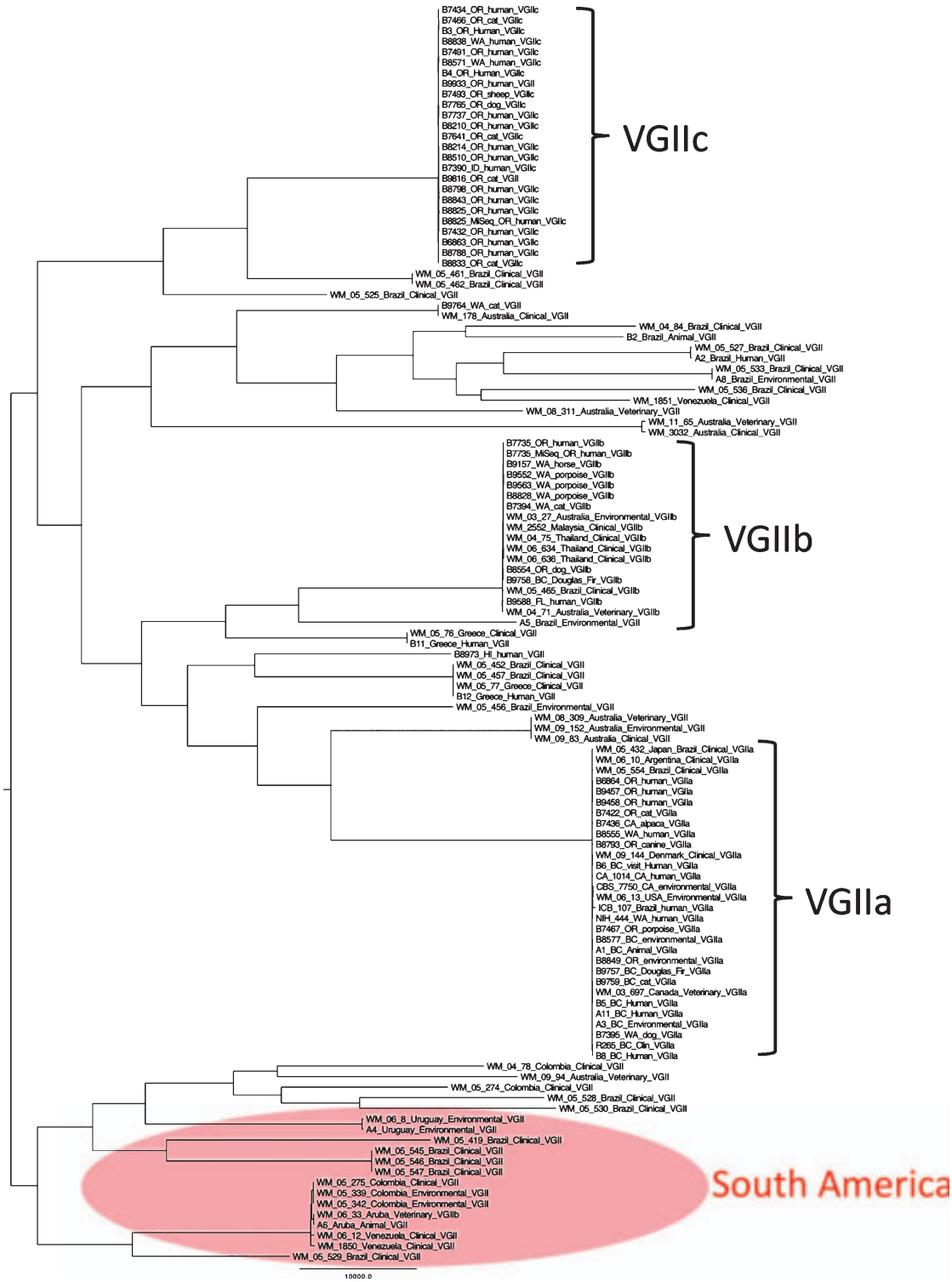

Figure 2. Single nucleotide polymorphism (SNP) phylogeny of *Cryptococcus gattii* molecular type VGII isolates with geographic annotation. Maximum parsimony phylogenetic analysis was performed on SNPs from 118 *C. gattii* VGII genomes including Pacific Northwest and global diversity. 310,969 SNPs were identified (221,248 parsimony informative). CI = 0.401.

currently being characterised to understand any functional role, by gene knockout studies carried out in our laboratory. WGS analysis showed again, that i) the highest genetic diversity within the VGII population is present in isolates from South America, ii) the major outbreak genotypes VGIIa and VGIIb are both present in South America, and iii) the Pacific Northwest genotype VGIIc is closely related to a South American isolate, which all support the evolutionary origin and dispersal of *C. gattii* from this part of the world (Figure 2)[12]. These findings were independently confirmed in a parallel study investigating 53 different VGII isolates[13].

Our current data indicate that Australia is not the origin of the North American outbreaks but a major stepping-stone in the global spread of outbreak-related *C. gattii* genotypes. As seen with the outbreaks in North America, *C. gattii* will continue to expand its ecological niche, because there is still a constant and dynamic process driving its evolution. Thus, further characterisation of isolates from regions like Australia, Brazil and Colombia, where a relatively high incidence of cryptococcosis due to *C. gattii* occurs in some native animals and indigenous human populations[3,4,14,15], need to be undertaken in order to better understand the key processes for the emergence of *C. gattii* epidemics around the globe, especially in regions where the yeast is not thought to be endemic.

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Biographies

Carolina Firacative was recently awarded a PhD from The University of Sydney and is now a postdoctoral fellow at the Molecular Mycology Research Laboratory, Westmead Millennium Institute. Her research focuses on the phenotypic and genotypic characterisation of clinically important fungal pathogens.

Dr Kennio Ferreira-Paim is a Post-doctoral fellow in the Molecular Mycology Research Laboratory at the Center for Infectious Diseases and Microbiology, Westmead Millennium Institute and a Biomedical Scientist at the Clinical Hospital of the Triangulo Mineiro Federal University, Uberaba, Brazil where he recently concluded his PhD in Tropical Medicine and Infectious Diseases. His research focuses on the molecular epidemiology of *Cryptococcus* spp. and studying the molecular basis of fungal virulence using gene knockout and reconstitution and animal virulence models. He is CAPES Science without borders visiting fellow (#9313133) from Brazil.

Dr Luciana Trilles is a Medical Mycologist working as researcher and curator of the Culture Collection of Pathogenic Fungi and professor in the Infectious Diseases Post-Graduation Course at the Infectious Diseases Institute, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil. Her research focuses on ecology, molecular epidemiology, molecular diagnosis and identification of systemic mycosis' agents.

Dr David M Engelthaler is the Director of TGen North, part of the non-profit Translational Genomics Research Institute, in Flagstaff, AZ, USA. David has over 20 years of public health research and practice history and was previously the Arizona State Epidemiologist and a biologist for the U.S. Centers for Disease Control and Prevention. He has published numerous papers on epidemiology, disease ecology, genetics, and microbiology.

The biography for **Professor Wieland Meyer** is on page 48.

On the surface of it: the role of materials science in developing antifungal therapies and diagnostics

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Surfaces are often considered to play a passive role in clinical mycology; that is, the outward face of a medical device to which fungal cells attach and form biofilms. However, materials chemistry and nanotechnology are now transforming passive surfaces into active interfaces and driving innovation into antifungal agents, their surface delivery and mechanisms, and diagnostic devices. Beyond technological improvements, there is great opportunity to drive basic research into fungal-surface interactions; however, this can only be accomplished with combined and concerted efforts of materials scientists, polymer chemists and mycologists.

Fungal biofilms on medical devices

Implanted devices are the source of 81% of nosocomial infections[1], costing the US $5–10 billion per year[2]. Fungal pathogens are the third most common cause of infections from catheters, leading to candidaemia[3], which has a mortality rate greater than 50% in Australia[4]. Particularly troublesome for treatment are fungal biofilms because they are pervasively established on surfaces through a process of rapid colonisation and spreading, followed by secretion of a protective extracellular matrix. Because of this, systemically administered antifungal drugs are nearly always ineffective at penetrating and eradicating a mature biofilm, necessitating the removal and replacement of infected devices. Clearly, new strategies are required for understanding the fungal-surface interface in order to reduce the unacceptably high rate of morbidity and mortality associated with infected urinary, central and venous catheters, endotracheal tubes, and other implanted biomedical devices.

Delivery from the surface: antifungal material coatings

Incorporation of antifungal agents onto the surface of medical devices in the form of surface coatings offers a novel way to prevent biofilm formation from the bottom up. This strategy allows concentration of antifungal agents at the material interface, killing potential colonisers or preventing them from attaching. Through the application of surface coatings, an active interface is furnished without altering the bulk properties of the substrate. Associated antifungal agents could take the form of currently licensed antifungal drugs or experimental compounds. There are broadly two types of coatings: (1) those that release a payload of antifungal agents, and (2) those where the active compound is irreversibly bound at the surface interface (Figure 1). The former comprise thin sponge-like coatings that are initially loaded with antifungal agents and become released into local tissues and fluids once implanted[5,6]. Releasing coatings with defined elution profiles would be suitable for short-dwelling implants in specific applications. The second strategy is to irreversibly bind or tether antifungal agents to the surface coating, providing a surface-contact effect without systemic release. Tethered antifungal agents would ideally utilise compounds with a known effect in the fungal cell wall (e.g. the echinocandins) that could act through a contact-killing mechanism. New research has shown this strategy to be remarkably effective against *Candida* spp. In our lab, we have prepared surface coatings with covalently attached caspofungin and demonstrated reduced surface attachment of 98% of *C. albicans* cells compared with untreated surfaces (unpublished data).

Moving from 2D to 3D: through the envelope

Tethering agents to the surface is a particularly attractive idea because it may be possible to formulate surface coatings using antifungal agents too toxic to be administered systemically. For some of the polyene class of drugs, which associate with ergosterol in the fungal cell membrane, many effective compounds have been developed but cannot be used because of their toxicity profile. One challenge that must be overcome using the tethering strategy is delivery of the agent to the cell membrane, which is protected by the thick cell wall. Using polymer surface grafting techniques it is possible to design nanoscale control of the linker with desired length, density and rigidity or fluidity. Such 3D approaches have successfully been used to study fibroblast adhesion by locating cell binding peptides onto and within so-called polymer brushes[7]. Beyond the delivery of antifungal agents, this technology could be harnessed to probe structures within the cell envelope to investigate changes in cell wall components that are important in morphogenesis and virulence[8]. This could be studied using live-cell imaging

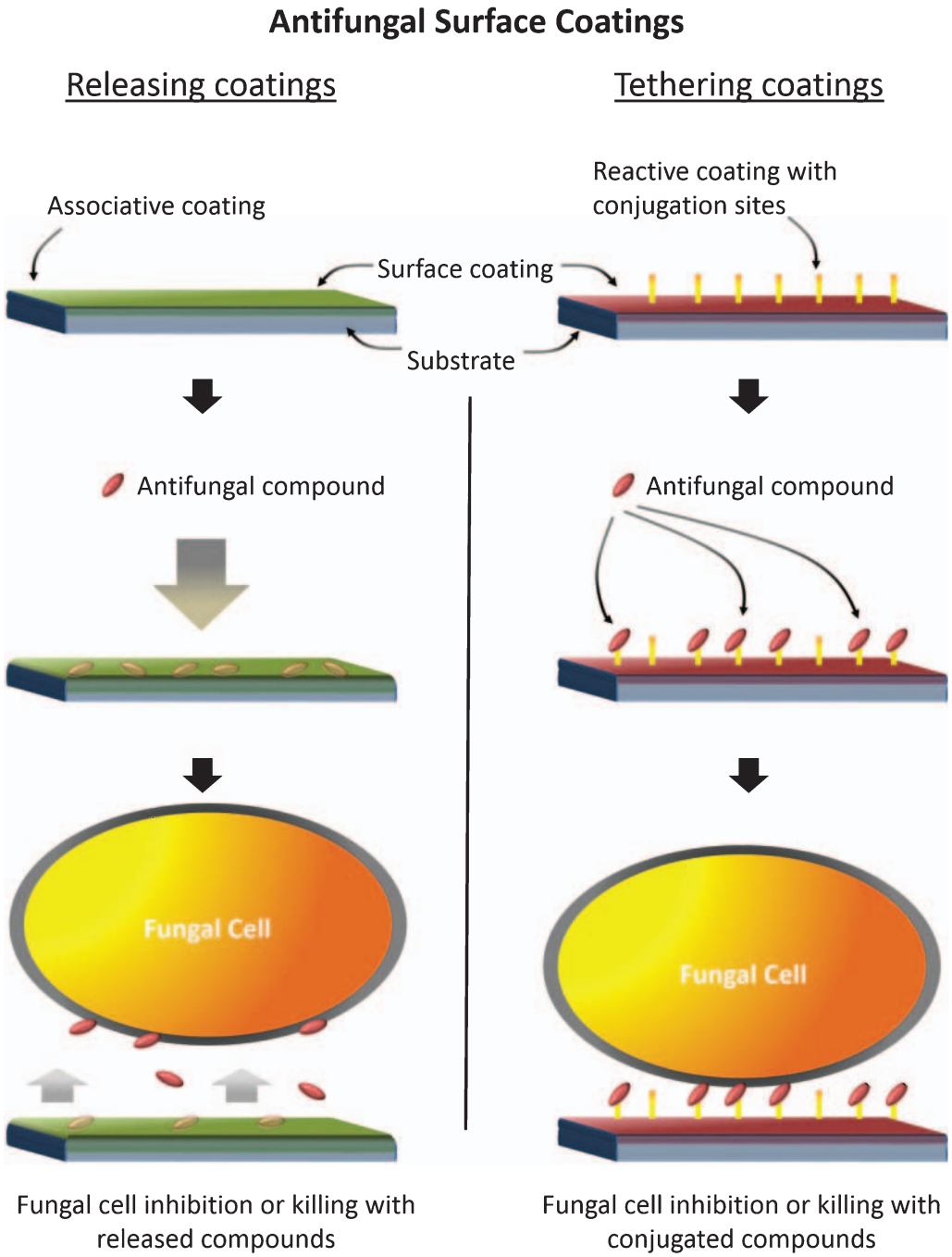

Figure 1. Two strategies for preparing antifungal surface coatings. Releasing coatings (left) incorporate antifungal drugs in an associative coating. Compounds become released and depleted on use. Tethering coatings (right) involve covalent attachment of the antifungal compound and act as contact killing surfaces.

techniques allowing real-time monitoring of morphological changes to the hyphal filaments[9]. Thus the combination of 3D grafting approaches with live-cell imaging provides a means to correlate the physical and chemical properties of the coating with observable changes to the structure and function of invasive filaments. This has potential to be a powerful method in pathogenesis studies. Furthermore, 3D grafting techniques that probe specific targets within the cell envelope will allow a more complete knowledge of cell wall structure and function, leading to a new understanding of drug mechanisms, organism pathology, and discovery of new diagnostic biomarkers.

Understanding mycology at the surface interface: new research, new opportunities

Breakthroughs in the biology of surface interfaces can only be accomplished by teams possessing an array of specialised skills: materials science, surface analysis, polymer chemistry, microscopy,

biochemistry, and cellular biology. Compared to anti-bacterial surfaces, progress has been slow in antifungal surfaces, evidenced by a 10:1 ratio of publications in these fields to date[10]. Clearly there is a disconnect between the importance of this topic to human health and the research outputs, despite calls by key opinion leaders for new strategies and therapies to combat invasive fungal infections[3,11] and labelling fungi as 'the unknown superbugs'[12]. The Mycology/Surface Interfaces Group is beginning to address these research gaps through basic research understanding of fungal-surface interactions. Part of this will feed into a greater understanding of clinically relevant poly-microbial infections[13]. Broader research will seek to apply discoveries to other areas including food and water safety, environmental moulds, and new diagnostic devices. Our long term goal is to develop partnerships with industries and translate research outcomes into innovative new products and therapies.

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Biography

Dr Bryan Coad is a Senior Research Fellow at the Mawson Institute, University of South Australia. His background is in physical chemistry and for the past 14 years has been active in the area of biomaterials design and surface analysis. He currently leads the Mycology/Surfaces Interfaces group. Recently, he was jointly awarded an Australian Research Council Discovery Project on combating fungal biofilm growth on surfaces.

Microsphaeropsis arundinis: an emerging cause of phaeohyphomycosis in cats and people





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***Microsphaeropsis arundinis* is an anamorphic dematiaceous fungus ubiquitous in soil and fresh water[1–4]. It typically inhabits terrestrial plant hosts[1–4] and has a well-known association with *Aruno donax*, a garden escape weed known as 'giant reed' or 'elephant grass'. *M. arundinis* (fungi imperfecti) is a coelomycete, which encompasses an emerging group of pathogens capable of causing soft tissue infections, mostly in immunocompromised human patients. Such disease typically arises secondary to traumatic inoculation of fungal elements into the subcutis. The infection may spread to contiguous subcutaneous tissues or via the lymphatics in a sporotrichoid manner. The first reports of this organism causing disease occurred just over 10 years ago, and since then an increasing number of cases have been encountered, but so far only in cats and people. In cats, lesions are most consistently encountered on their distal extremities, viz. on or near the toes.**

In 2004, Kluger *et al.* reported the first *Microsphaeropsis arundinis* infection in a mammalian host[1]. The patient was a seven-year-old cat living in suburban Sydney. It had a granulomatous lesion within the deep tissues of the distal forelimb. The cat had a concurrent *Fusarium chlamydosporum* infection affecting another limb. A few months later, Pendle *et al.* from Royal North Shore Hospital reported the same organism as a cause of disease in two immunocompromised human patients, with limited archival information on a third case, a patient with acute myeloid leukaemia seen 23 years earlier[2]. Is it a coincidence that the first reports of a new mammalian fungal pathogen occurred at virtually the same time, and in the same city, in both human and veterinary (feline) patients? It may be, but it would neglectful not to look further for factors that may explain why humans and cats were becoming infected by this hitherto non-pathogenic fungus. These events also emphasise the 'One Medicine – One Health' approach to infectious disease investigation, with animals representing sentinels for the occurrence of human disease. This is particularly the case for fungal diseases acquired from the environment.

In 2009, the first *M. arundinis* infection in the USA was reported in a human patient receiving immunosuppressive therapy for

a renal transplant[3]. The man was domiciled in Florida, an area with a subtropical environment likely favourable to this organism. Sydney, while potentially temperate in climate by latitude, is classified as subtropical in rainfall distribution and temperature, with summer distribution of rainfall and mild winter temperatures.

In 2010, our group again reported disease caused by *M. arundinis* infection affecting the distal extremity of a cat (Figure 1), although this animal also had a lesion on its face[4]. Halliday molecularly characterised the internal transcribed spacer (ITS1), *5.8S* and ITS2 regions and the D1/D2 region of the *28S* rDNA gene cluster of the available human (four) and feline (two) isolates[4,5]. These included a case (contributed by Tom Gottlieb) of refractory dermal plaques in a renal transplant recipient (Figure 2; Table 1). The isolates were deposited in various culture collections and the merged ITS and LSU sequences of the six isolates were deposited in the GenBank database (www.ncbi.nlm.nih.gov/genbank/).

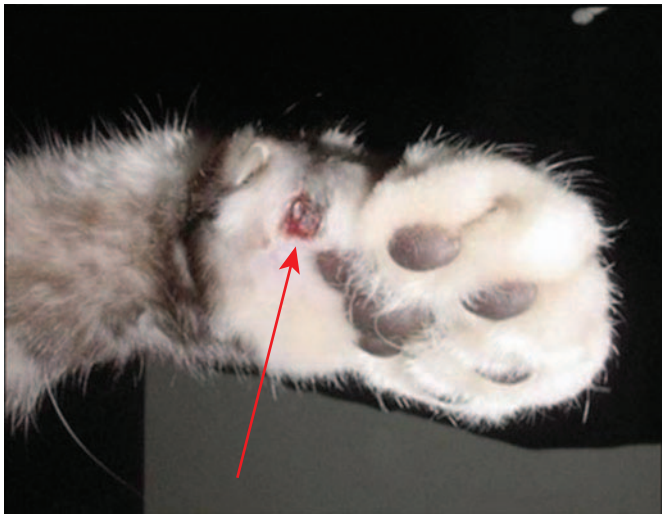

Figure 1. Appearance of a localised *Microsphaeropsis arundinis* infection affecting the interdigital web of a cat. The lesion is highlighted by a red arrow.

Since the first human report by Pendle *et al.*[2], there have been at least six additional *M. arundinis* infections reported in human patients, individual cases being seen at St George Hospital, Wollongong Hospital, Concord Hospital (Figure 2), Westmead Hospital and Prince of Wales Hospital, and a further case from Florida in the USA (Table 1). The additional five Australian cases have been collated and submitted for peer review, including the case in Table 1 and Figure 2.

In the veterinary arena, we continue to see *M. arundinis* infections in cats along the East coast of Australia. It is now probably the most common cause of feline subcutaneous phaeohyphomycosis in this region, with five additional cases between 2009 and 2012 (Table 2). There does not appear to be any age predisposition in cats (range

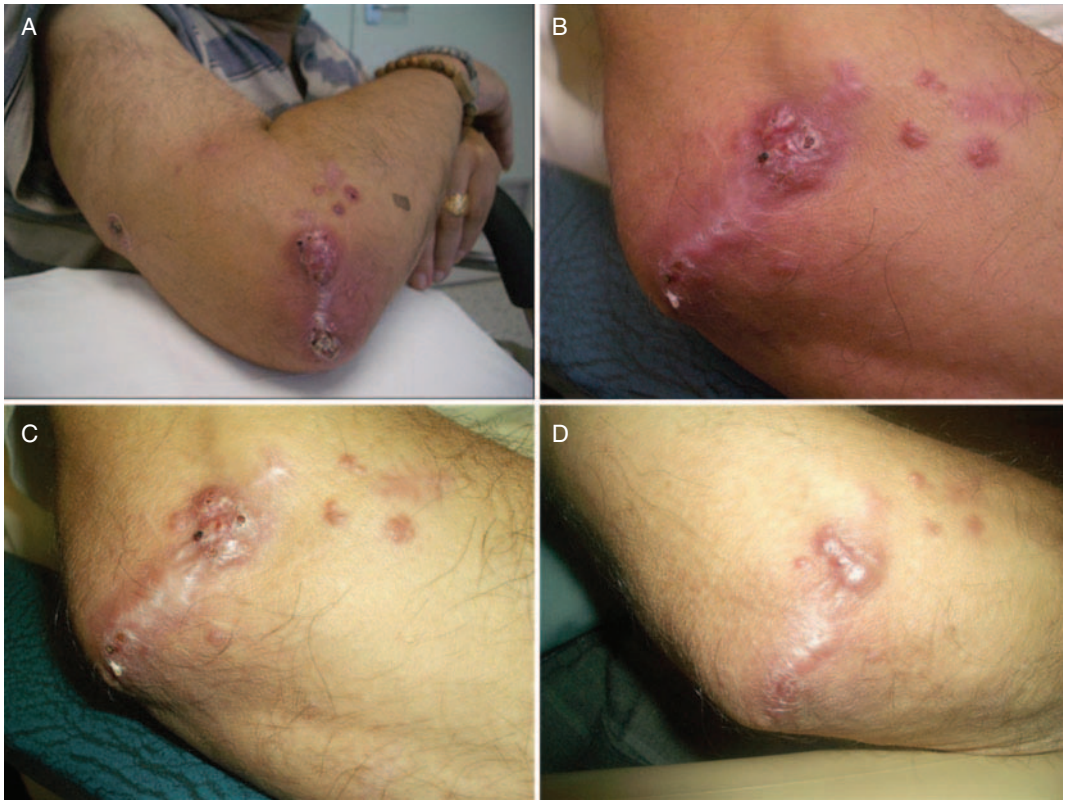

Figure 2. Localised *Microsphaeropsis arundinis* infection on the right elbow of a renal transplant recipient. The infection developed after the patient fell on concrete. The initial lesion is shown in A, while B, C and D show progressive improvement during itraconazole therapy. Note the smaller satellite lesions in the vicinity of the large primary lesion, suggesting sporotrichoid spread via the lymphatics.

Table 1. Summary of all human *Microsphaeropsis arundinis* infections for which we have information (1981–2014).

Date reported	Case number[A]	Age (years), gender	Lesions	Co-morbidities	Cytology or histology findings	Culture or PCR results from any culture	Treatment	Outcome
2004	Case 1 Pendle *et al.* (2004)[2] Sydney, Australia	80, M	3 cm diameter painless plaque on dorsum of right hand	Non-insulin dependent diabetes and long term corticosteroid therapy	Irregular septate hyphae	*Microsphaeropsis arundinis*	Terbinafine for 3 months	Lesion resolved over 20 weeks (after starting therapy) and did not recur
2004	Case 2 Pendle *et al.* (2004)[2] Sydney, Australia	56, M	Ulcers on both feet	Chronic renal failure, ankylosing spondyloarthropathy treated with corticosteroids	Irregular septate hyphae	*Microsphaeropsis arundinis*	Itracoanzole for 10 months and amputation of gangrenous toes	Eventually resolved after 10 months
1981	Case 3 Mentioned in Discussion of Pendle *et al.* (2004)[2] Sydney, Australia	N/A	Ankle nodule	Acute myeloid leukaemia	Leukaemic infiltrate and fungal elements	*Microsphaeropsis arundinis*	N/A	N/A
2013	Case 4 Hall *et al.* (2013)[3] Jacksonville, Florida, USA	70, M	Crusted, ulcerated plaque on the dorsum of the right middle finger	Renal transplantation	Hyphae	*Microsphaeropsis arundinis*	Posaconazole for 6 months	Clearance of the lesion over 6 months
2002	Case 5 Gottlieb and colleagues Concord Hospital	55, M	Irregular plaques on dorsum of the right elbow (Figure 2) after a fall on concrete	Renal transplantation	Irregular septate hyphae	*Microsphaeropsis arundinis*	Itracoanzole for 11 months	Clearance of the lesion over 11 months

[A]The authors are aware of three additional cases in humans from 2013 to 2014, but for which details are unavailable as the manuscript is currently under peer review.

5–20 years) and no gender preponderance. Geographically, two cats were domiciled in Sydney, two cats resided in the Central Coast of NSW, one was from Newcastle, another from Wollongong, while the last cat was from Brisbane. While there is the potential of geographical bias due to the catchment area of our pathology services, all these cats lived in coastal environments, which are becoming increasingly warmer and more humid. Lesions were invariably present on distal extremities, with either forelimbs or hind limbs being affected. Microscopy of needle aspirates or crush preparations from lesions were suggestive of phaeohyphomycosis, with pigmented bulbous septate hyphae or pseudo-hyphae of variable length, and occasional yeast-like forms evident in the tissues (Figure 3*B–D*). Histopathological specimens showed pyogranulomatous inflammation (Figure 3*A*), with occasional multi-nucleate giant cell formation. The organism grows well on routine fungal media such as Sabouraud dextrose agar (containing antibiotics) and microscopically shows irregularly-shaped pigmented septate hyphae, but no conidia. Since species identification is made difficult by the inability to induce sporulation, PCR amplification and sequence analysis was typically used to establish a specific diagnosis (as outlined above). This could be done using not only colonial material or fresh biopsy specimens from representative lesions but also paraffin-embedded formalin-fixed tissues[5]. Unlike the situation in people where most patients appear to be immunosuppressed by comorbid disease (renal failure, diabetes), corticosteroids or immunomodulatory drugs, most cats appear immune-competent. Cats with co-infections with other fungi (e.g. *Fusarium* spp.) are postulated to have had penetrating injuries contaminated by multiple fungi normally residing in soil.

In veterinary practice, frustratingly, repeat samples for culture and antifungal susceptibility are often difficult to obtain. This is usually because serial specimen collection generally requires sedation or anaesthesia with concomitant cost and morbidity. To overcome this potential limitation, we have found that there is sufficient fungal nucleic acid preserved in methanol-fixed, DiffQuik®-stained smears to permit successful DNA purification, panfungal PCR and sequence analysis using material scraped from the cytological specimens (Table 2). The method[6], adapted from a similar technique used for diagnosis of veterinary mycobacteria specimens[7], was successful in 4/4 feline *M. arundinis* cytology slides in which it was attempted (Table 2).

Antifungal minimum inhibitory concentration breakpoints have not been determined for this organism, although broth microdilution assays suggest most isolates are susceptible to broad spectrum azoles (itraconazole, voriconazole and posaconazole), amphotericin B and terbinafine, with variable susceptibility to fluconazole and resistance to echinocandins and flucytosine. Clinically, itraconazole, posaconazole, ketoconazole and terbinafine all appear to have good *in vivo* activity.

As voriconazole causes neurotoxicity in many feline patients[8], we currently recommend posaconazole to treat *M. arundinis* infections in cats. Although the drug is expensive, it is palatable, available in a liquid form, has minimal hepatotoxicity (unlike itraconazole) and possesses favourable and reliable pharmacokinetics in this species, where once daily administration with meals is convenient for owners[4]. Terbinafine is probably also a good option for cats, with established pharmacokinetics and inexpensive generic

Table 2. Summary of all feline *Microsphaeropsis arundinis* infections for which we have information (2001 to 2014).

Date	Case number (reference)	Australian postcode/locality	Age (years)	Main clinical findings	Cytology/histology findings	Microbiological findings	Specimen submitted for PCR	CIDMLS Westmead ICPMR panfungal PCR test result
2001	Case 1 (Kluger *et al.* 2004[1])	2124 Parramatta, NSW	7 MN DSH	Focal abscess-like lesion left proximal interphalangeal region (toe)][A] [NB: concurrent infection with *Fusarium chlamydosporum*]	Large spherical bodies to septate branched hyphae and chains of smaller yeast-like cells	Fungal culture: non-sporulating dematiaceous fungus	1 plate	*Microsphaeropsis arundinis*
2004	Case 2 (Krockenberger *et al.* 2010[4])	2234 Menai, NSW	11 FN DSH	Swelling of subcutis over bridge of nose and focal swelling near the dewclaw pad of the left front limb[B]	Spherical fungal elements	Fungal culture: non-sporulating dematiaceous fungus	1 plate	*Microsphaeropsis arundinis*
22 Apr 2009	Case 3	2258 Ourimbah, NSW	5 FN DSH	Raised 2 cm diameter non-painful soft tissue swelling right hind limb in lateral metatarsal/phalangeal region	Numerous bulbous septate fungal hyphae	*Microsphaeropsis arundinis* or *Paraphaeosphaeria* sp.	1 smear	*Microsphaeropsis arundinis*[C]
30 Jul 2010	Case 3 (cont.)	2258 Ourimbah, NSW	5 FN DSH	Foot swelling (recurrence)	Numerous bulbous septate fungal hyphae	*Microsphaeropsis arundinis* or *Paraphaeosphaeria* spp.	1 smear	*Microsphaeropsis arundinis*
13 Jan 2006	Case 4	2500 Wollongong, NSW	8	Soft, non-painful haired mass lateral to digit 3 left hind leg Present for approximately 3 weeks	Pyogranulomatous inflammation with numerous fungal hyphae	Fungal culture: no growth	1 smear	*Microsphaeropsis arundinis*
18 Aug 2012	Case 5	2305 New Lambton, NSW	14 FN DSH	Non-painful swelling left forelimb digit 3. Present for 2–3 weeks	Numerous septate fungal hyphae of variable length and yeast-like forms	Not cultured	2 smears	*Microsphaeropsis arundinis*
28 Mar 2012	Case 6	2259 Tuggerah, NSW	9 MN DSH	Interdigital draining sinus tract affecting right front paw [NB: this patient had prior fungal disease of right hind limb caused by *Fusarium* spp. in 2010]	Cytology: abundant fungal hyphae Histology: subcutaneous mycosis	Fungal culture: dematiaceous fungus	1 plate	*Microsphaeropsis arundinis*
4 Sep 2012	Case 7	4122 Mt Gravatt, Qld	20 MN DSH	Toe lesion	Subcutaneous mycosis	Fungal culture: dematiaceous fungus	1 plate	*Microsphaeropsis arundinis*

[A]Infection recurred 2.5 years after treatment with ketoconazole and the toe was amputated.
[B]Persistent recurrence.
[C]S to Amphotericin B, Itraconazole, Voriconazole and Posaconazole; R to Fluconazole.
DSH, domestic short-haired cat; FN, female neuter (spayed); MN, male neuter (castrated).

formulations available, but we have no experience to date. Despite long courses of therapy, and in some cases cytoreductive surgery (typically toe amputation), there is a tendency for infections in cats to recur months or years after apparently successful therapy. This does not appear to be the case in human patients. Despite this propensity to recur, the organism seems inherently of low virulence, producing indolent infections without dissemination. This is possibly due to the organism favouring lower temperatures for growth, like many fungal saprophytes. The few well documented human cases reported to date have been cured with monotherapy using a triazole or terbinafine, sometimes combined with debridement or amputation of infected tissues[2,3].

We speculate that this infection might be becoming more common due to expansion in the range of 'elephant grass' or expansion of other plant hosts capable of supporting its environmental growth. The location of lesions on the toes of cats suggests penetrating injury of distal extremities during digging may play a role in disease pathogenesis. For the same reason, it might be expected that cat scratch injuries (typically to the face) might also be contaminated by this organism[9,10], as in the case reported by Krockenberger *et al.*[4]. Similar predisposing factors may be operating in people who garden without protective measures (gloves and long sleeved apparel) and therefore may be at risk for development of *M. arundinis* infections through contamination of skin wounds, especially if they have diabetes or are receiving immunosuppressive therapy. It is interesting that cats have an apparent predisposition, as to date there have been no reported infections in non-feline veterinary patients, i.e. dogs, horses, cows, etc. The reason for this species predilection requires investigation. Time may reveal the answers to these questions as we actively search for more cases!

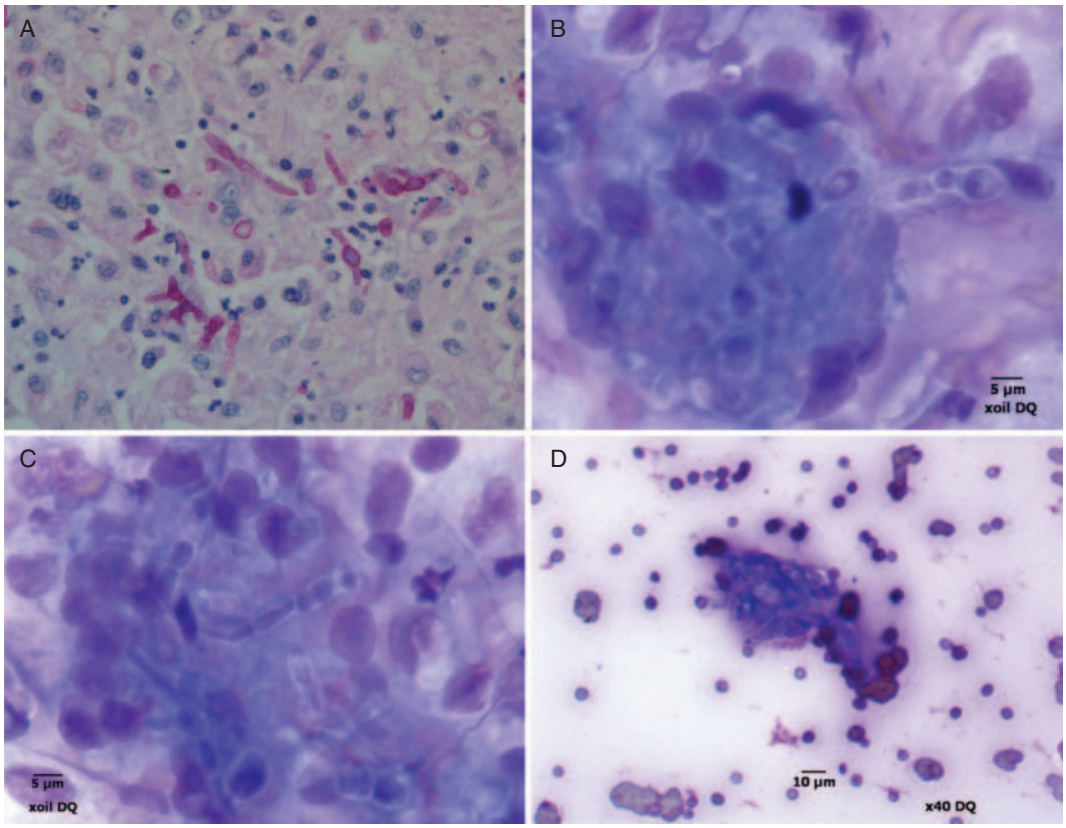

Figure 3. Representative microscopic appearance of *Microsphaeropsis arundinis* in periodic acid Schiff (PAS)-stained histological sections (A) and Diff-Quik®-stained smears from aspirates (B–D) from lesions on the distal extremities of cats. The hyphae have obvious septation, are of variable length, with occasional globose dilatations and yeast-like forms visible in different parts of the smear.

Acknowledgements

The authors thank Sharon Chen for information on the additional Australian human *Microsphaeropsis arundinis* infections from a manuscript currently under review.

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Biographies

The biography for **George Reppas** is on page 82.

Associate Professor Gottlieb is head of Infectious Diseases at Concord Hospital, and a clinical senior lecturer at the University of Sydney. He is the current president of the Australian Society for Antimicrobials (ASA) and immediate past president of the Australasian Society for Infectious Diseases (ASID). He is on the executive of the Australian Group on Antimicrobial Resistance (AGAR). He represents ASA on the Antimicrobial Resistance Standing Committee reporting to the Australian Government. He has been chair of advisory committees supervising training in Infectious Diseases and Microbiology, and has participated in the writing groups for the Australian Infection Control Guidelines and Therapeutic Guidelines for Antibiotic. His interests include the diagnosis and management of clinical infectious diseases, hospital infection control and antimicrobial resistance.

The biography for **Dr Mark Krockenberger** is on page 82.

The biography for **Catriona Halliday** is on page 82.

The biography for **Richard Malik** is on page 82.

Pneumocystis canis pneumonia in dogs





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Pneumocystis canis **is a potential cause of life-threatening interstitial fungal pneumonia in dogs. It is seen almost exclusively in two canine breeds, miniature Dachshunds and Cavalier King Charles Spaniels (CKCS)[1]. Historically, Australian veterinarians had a key role in the documentation of this entity and its conspicuous breed associations[2–4]. Affected Dachshunds and CKCS are likely to have an inherited immunodeficiency that predisposes them to infection with this commensal organism of the respiratory tract and pharynx[1,2,5,6]. A high index of suspicion is required to make a timely diagnosis and save affected patients, as these dogs cope poorly with anaesthesia and other measures to procure the specimens required to make a definitive diagnosis. Possible co-infection with** ***Bordetella bronchiseptica*** **must be considered when determining antimicrobial strategies. Affected dogs occasionally have a previous or concurrent history of generalised demodicosis[5,7,8]. With early intervention, affected dogs can be saved, although some require lifelong therapy to prevent recurrence. The future challenge is to develop fast molecular techniques to diagnose** ***P. canis***[†] **pneumonia (PCP)[7,9] and to determine the underlying immune defect in over-represented breeds through the rapidly advancing field of canine genomics[10].**

Pneumocystis spp. are ubiquitous commensals of the respiratory tract of many mammalian species, including dogs[1]. This group of organisms has the potential to cause life-threatening pneumonia in a wide range of mammals, including rats, pigs, horses and goats[1]. *P. jirovecii* has the same pathogenic potential in people[1]. Airborne droplet transmission from subclinically affected normal dogs, often transmitted from bitch to pup soon after birth, is suspected of being the means for transmission[1]. PCP results from the effects of masses of organisms within the alveolar spaces, combined with the associated inflammatory response of the host[1–6].

Historically, most canine cases had been reported in young (less than one-year-old) miniature Dachshunds[1–3,6,11]. The first documentation of this entity was provided in a paper from the University of Sydney (UoS) by Farrow and colleagues[2], although a brilliant

[†]The literature refers to *Pneumocystis carinii* infection of dogs, although recent taxonomic developments suggest this name should be probably restricted to organisms that infect rats. One conference Abstract suggests the name *Pneumocystis canis*[9], although *Pneumocystis* special form *canis* may be a safer term in the interim. For the purposes of this article we have used the term *P. canis* for simplicity.

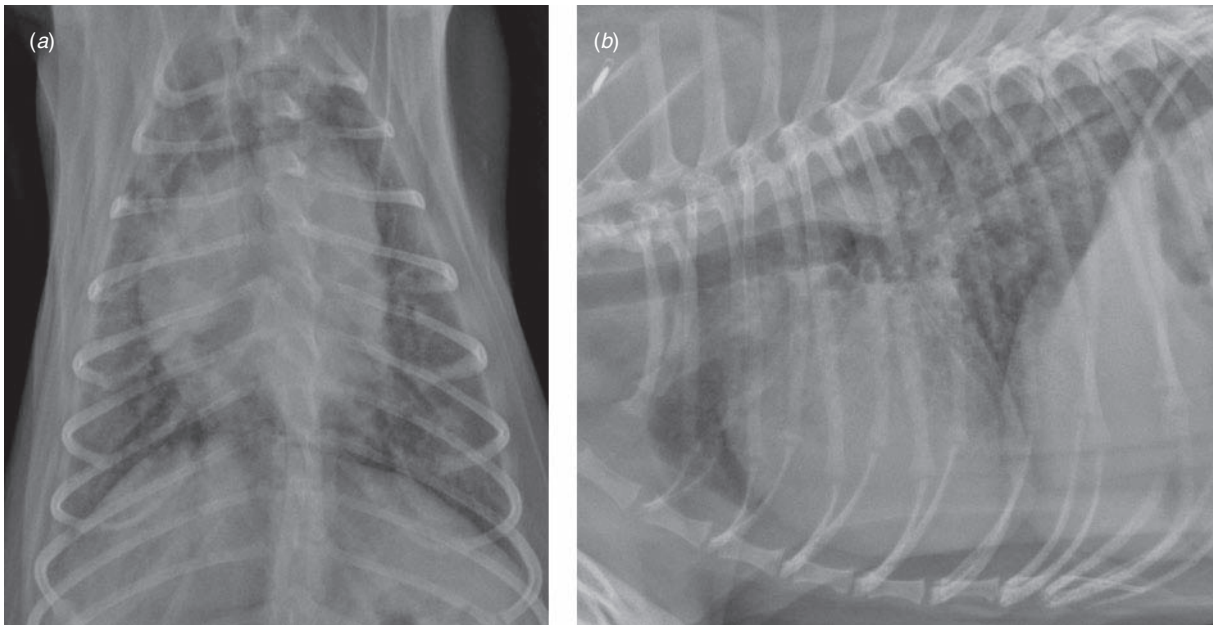

Figure 1. (*a*) Dorsoventral thoracic radiograph from a one-year-old Cavalier King Charles spaniel with pneumocystosis, revealing a patchy, diffuse, interstitial pattern in all lung fields. (*b*) Right lateral thoracic radiograph of the same dog reveals a diffuse interstitial to alveolar pattern, especially in the dorsocaudal lung fields.

immunoparasitologist at McMaster Laboratory (Robert J. Love) provided key immunologic insights.[‡] The disease in Dachshunds was characterised further by South African veterinarians, with Lobetti and colleagues adapting human treatment strategies to successfully manage their canine cases[1,11]. The best radiological description of PCP was provided by Kirberger[12], although this has been complemented by recent advances in imaging such as digital radiology and computed tomography (CT). Although PCP continues to be seen in Dachshunds in South Africa and the USA, the great majority of cases encountered in Australia over the past 20 years have been in adult CKCS, the first detailed report being provided by Paul Canfield and colleagues from UoS in 1993[4]. This breed preponderance was then detected in the UK[5,13] and subsequently Europe, the USA and Japan[7]. 'Cavaliers' tend to get the disease as young adult dogs[4,5,13]. There may be antecedent or concurrent footprints of immune deficiency, such as generalised demodicosis[5,8].

In people, the disease is best known as a complication of HIV/AIDS, although it is also seen in transplant recipients and other patients receiving immunosuppressive drug regimens. A very well characterised case cluster in Australia that closed a transplant ward at Westmead Hospital was reported by Sharon Chen, Wieland Meyer and their ANZMIG colleagues[14]. In Arabian horses and related breeds, foals with autosomal recessive severe combined immunodeficiency syndrome (analogous to the like-named SCID condition of men and mice) typically died of PCP in the neonatal period[15], and this was common in the Camden district in the 1970s. A test was developed for this genetic disease of horses, and nowadays the condition is hardly encountered, a cogent example of genetic counselling and preventative veterinary medicine[16].

In dogs, the nature of the immune defect in Dachshunds and Cavaliers has not been determined at the molecular level. Poor lymphocyte stimulation (despite normal lymphocyte numbers in peripheral blood) has been documented in miniature Dachshunds[1,2]. IgA, IgM and IgG concentrations are subnormal in affected members of this breed[6]. Subsequent assessment of CKCS with pneumocystosis also reveals immunoglobulin deficiencies and decreased lymphocyte function[5,7]. These immunodeficiencies persist after resolution of the infection with effective antimicrobial therapy. Studies of the involvement of adaptive cell-mediated immunity and innate immunity (including the potential involvement of Toll-like receptors) would be sensible but have not yet been performed.

Most dogs with PCP present with respiratory signs, including dyspnoea, tachypnoea, increased breath sounds on thoracic auscultation or cyanotic mucous membranes. The presence of a cough is variable, and this sign is frequently absent. Duration of signs prior to presentation for veterinary attention can be as long as four weeks[1–4]. Haematologic and serum biochemical abnormalities are non-specific[1]. Thoracic radiographs reveal diffuse, bilaterally symmetrically increased radiodensity of the pulmonary parenchyma, classically described as a miliary-interstitial to alveolar pattern (Figure 1)[1,12,17].

[‡]The first report of PCP pneumonia in dogs was actually from Germany in 1955. The patient was a nine-week-old sheepdog pup with *Pneumocystis* forms in its lungs, hilar lymph nodes and myocardium. Sedlmeier, H. and Dahme, E. (1955) *Pneumocystis carinii*-Infektion biem Hund. *Zentralbl. Allg. Path.* 93, 150–155.

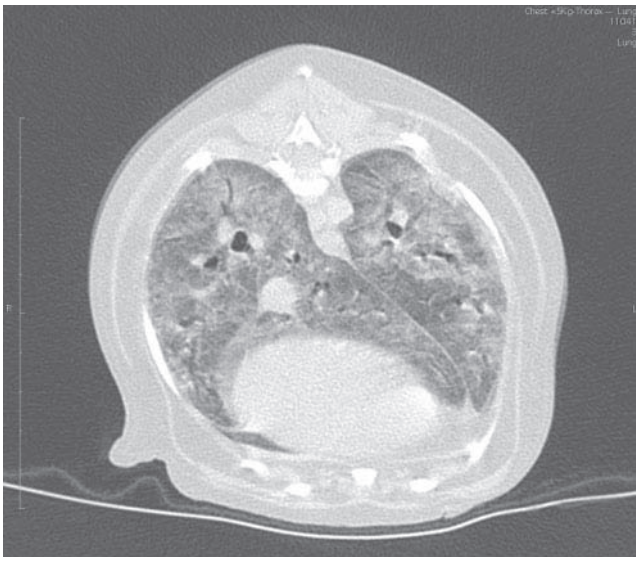

Figure 2. Thoracic computer tomography scan in the same dog as Figure 1 reveals a marked, patchy, increased pulmonary opacity throughout all lung lobes with some peripheral consolidation. The ground-glass appearance of the lung parenchyma is quite characteristic of advanced PCP in the dog.

There is often radiological evidence of right-sided heart enlargement and pulmonary hypertension, and this can be confirmed echocardiographically[4]. Solitary opacities, cavitary lesions or a non-symmetric radiographic changes are less commonly observed[1,12]. Severe long-standing cases may develop emphysema[1]. The CT findings in canine PCP are dramatic (Figure 2), providing a better appreciation of the lung pathology and disclosing regional lymphadenomegaly.

Diagnosis can be challenging in a veterinary context, with identification of *P. canis* 'cysts' in bronchoalveolar lavage fluid (BAL) or transthoracic fine needle aspirates of lung generally being required. Intact cysts are distinctive, being 5–10 µm in diameter and containing 4–8 dark-staining intracystic bodies (2–3 µm in diameter; Figure 3). PCR testing of BAL fluid or lung aspirates is uncommon in veterinary laboratories, but should probably be attempted more often[7,9], as the requirement to perform open lung biopsies to secure a diagnosis is unnecessarily invasive given the characteristic nature of the imaging findings and the strong breed associations. If BAL fluid specimens are obtained, they should be subjected to routine bacterial culture as well as microscopy and PCR, as *Bordetella bronchiseptica* can be an important co-morbidity in canine PCP pneumonia. If a BAL is not done and treatment is based on a presumptive diagnosis, then doxycycline should be given as well as trimethoprim sulfonamide to cover this possibility. Research is currently focused on development of qPCR assays for canine *P. carinii* strains, as assays developed for *P. jirovecii* may not detect *P. canis* associated with infections in dogs[1]. Panfungal PCR with sequence analysis provides an interim molecular diagnostic strategy, as does genus-specific *Pneumocystis* PCR assays[7–9].

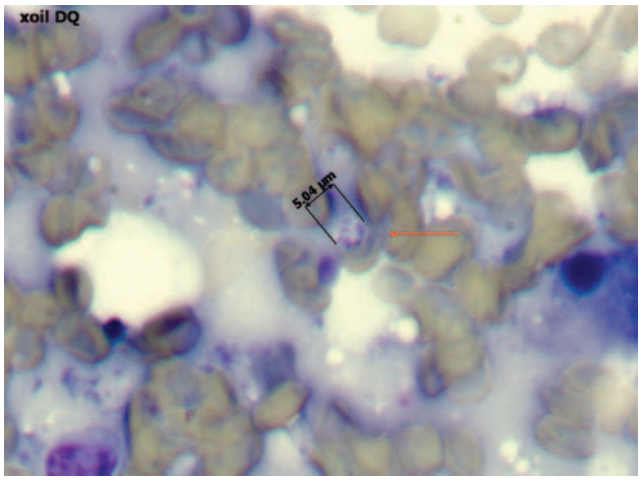

Figure 3. A single cyst-like structure (red arrow) suspicious of *Pneumocystis spp.* 'cyst' seen on cytology from a one-year-old Cavalier King Charles Spaniel with pneumocystosis (Diff Quik® stain, oil immersion microscopy).

Treatment includes appropriate antimicrobial therapy, using intravenous trimethoprim sulfonamide combinations, and less commonly nebulised pentamidine, along with appropriate supportive care (e.g. supplementary oxygen, nebulisation, chest physiotherapy). Long-term ventilation of affected dogs is challenging and beyond the financial limits of most owners. There is no zoonotic potential for humans in close contact with a *Pneumocystis* infected dogs, as the organisms in humans and dogs are distinct and no evidence of cross-species transmission has been documented.

As thoughtful and responsible veterinary physicians, we should be banking DNA from all dogs with confirmed and presumptive PCP. The most likely way to eliminate this condition is by harnessing the power of canine genomics to detect any underlying genetic defect, utilising either a genome-wide association study or whole genome sequencing of 'Trios' of affected and closely related dogs[10]. It is much better to try and prevent this severe mycosis by screening the breeding population using a molecular genetic test, rather than to have to treat a severely affected individual. This is where vets have an advantage over medics, we can use eugenics!

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Biographies

Elizabeth Ralph is a veterinarian and has recently finished her residency in small animal internal medicine and is well on the way to her goal of becoming a small animal internal medicine specialist. Elizabeth has also worked with the Department of Primary Industries during the Equine Influenza outbreak in 2007 and with her local government as a member of the Companion Animal Advisory Committee. Her professional interests include small animal internal medicine, especially immune-mediated disease.

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Mucormycosis in the platypus and amphibians caused by *Mucor amphibiorum*

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Mucormycosis in the platypus and the anuran (frogs and toads) is a serious fungal disease affecting these aquatic taxa. *Mucor amphibiorum* infection causes significant morbidity and mortality in free-living platypuses in Tasmania. Infection has also been reported in free-ranging cane toads and frogs from mainland Australia, but not confirmed in platypuses from the mainland. This paper reviews mucormycosis in the platypus and anuran, including consideration of the clinical, epidemiological, pathological and diagnostic features.

Mucor amphibiorum

Mucor amphibiorum is a dimorphic fungus in the Mucorales order of the Zygomycetes class of fungi. Its sporangiospores, when found in infected tissues, occur as the yeast form (spherule-like structures, containing 2–11 daughter spherules) or develop into the more usual non-septate hyphal form on culture media or in the environment[1,2]. Infections (Table 1) have been reported in a range of anurans (frogs, toads), and the platypus[1,3,4,6,8–10]. Transmission between captive anurans and salamanders has been documented; while experimentally infected reptiles remained clinically healthy with only small lesions at necropsy, and no lesions were reported in

Table 1. Hosts reported with *Mucor amphibiorum* infection globally.

Scientific Name	Common Name	Geographical origin and/or place held	Reference
Aparasphenodon sp.	Casque-headed frogs	South America (captive in Germany)	1
Bufo bufo	Common toad	Europe (captive in Germany)	1
Rhinella marina (Bufo marinus)	Cane toad	QLD & NT, Australia (free-living)	3
Dendrobates sp.	Poison arrow frog	South America (captive in Germany)	1
Limnodynastes peronii	Striped marsh frog	Australia (free-ranging)	4
Litoria adelaidensis	Slender Tree Frog	Australia (captive in Perth Zoo)	5
Litoria caerulea	Australian green tree frog	Australia (captive in Germany)	6
Litoria caerulea	Australian green tree frog	QLD, Australia (free-living)	4
Litoria infrafrenata	White-lipped tree frog	Australia (captive in Melbourne Zoo)	7
Litoria infrafrenata	White-lipped tree frog	Australia (captive in Perth Zoo)	5
Ornithorhynchus anatinus	Platypus	TAS, Australia (free-living)	8, 9, 10
Rana temporaria	European common brown frog	European (experimental infection)	6
Rana esculenta	Edible frog	Europe (experimental infection)	6
Salamandra salamandra	Fire salamander	Germany (Captive)	1
Trachycephalus sp.	Milk frog	South America (captive in Germany)	1

QLD, Queensland; NT, Northern Territory; TAS, Tasmania.

experimental infections of laboratory animals[1,6]. *Mucor amphibiorum* appears to be endemic in Australia, infecting free-living frogs and toads in Queensland, New South Wales and Northern Territory, with accidental introductions into captive frogs in Melbourne and Perth[5,7] and platypuses in Tasmania. It seems unlikely that it was introduced into Australia with cane toads in 1935, as Speare *et al* (1994)[3] was unable to isolate it from 41 cane toads sampled in Hawaii or Costa Rica.

Mucormycosis in anurans

Mucor amphibiorum was first reported from a German Zoo in 1972, where it resulted in disseminated disease in a common green tree frog (*Litoria caerulea*) imported from Australia, and subsequently in frogs, toads and salamanders in neighbouring exhibits[1,6].

In amphibians, mucormycosis caused a disseminated disease with multiple white nodules in liver, kidney, bladder and lung, the emaciated animal dying within 2–4 weeks. Skin involvement via lymphatic spread was observed in 42% of infected toads, but skin ulcers were rare[11]. Histologically, nodules consisted of granulomas and pyogranulomas containing thick-walled spherules (5–37 µm diameter, containing 0 to 10 daughter spherules). Of nine *M. amphibiorum* isolates from cane toads (*Rhinella marina*), five were positive mating strains and four were negative mating strains[11]. The route of entry of *M. amphibiorum* in the anuran is likely ingestion of soil contaminated by faeces excreted by infected animals. *M. amphibiorum* was isolated from 2/20 soil specimens from an endemic site in Townsville where resident cane toads had mucormycosis. Furthermore, the organism has been shown to grow and sporulate in soil[11].

Figure 1. Gross appearance of mucormycosis in the Tasmanian platypus. (*a*) Severe chronic ulceration of left hind leg, with granulation tissue encircling leg and spur. (*b*) Ulceration of the dorsal tail (60 x 43 mm), with thickened edges and central cavitation. (*c*) Chronic ulceration of right hind leg (100 x 40 mm), with serous exudate and bleeding. (*d*) Hairless raised nodules on tail, some full thickness and exuding pus (bar = 10 mm).

Mucormycosis in the platypus

Munday and Peel (1983)[8] first described four cases of ulcerative dermatitis in dead and debilitated Tasmanian platypuses from the Elizabeth River in Campbell Town, but the causative agent was not identified as *M. amphibiorum* until 1993[9]. *M. amphibiorum* causes a severe granulomatous and often ulcerative dermatitis in the platypus, which may progress to involve underlying muscle and occasionally disseminate to internal organs, particularly the lungs[9], leading to death. In the absence of the systemic spread of the organism, death can also result from secondary bacterial infections or impaired thermoregulation and mobility.

All 17 platypuses with mucormycosis captured during a 12 month Tasmanian study[10] were alert and displayed normal responses to capture and handling. Gross appearance of skin lesions varied from non-ulcerated, hairless nodules and abscesses, to ulcers with undermun or thickened margins, sinuses exuding pus, or exuberant granulation tissue (Figure 1). Some lesions appeared as discrete entities. Others coalesced to form plaques. Lesions were found on haired regions including the hind limbs (38%), forelimbs (6%), tail (19%), trunk (6%) and head (6%), and unhaired regions such as the webbing of the forelimbs (13%) or bill (6%). Some affected animals had lesions at more than one site. One platypus had a tail ulcer which reduced in size over a three month period. Of 13 isolates of *M. amphibiorum* from 17 diseased platypuses, all were of the positive mating strain[10]. One platypus *M. amphibiorum* isolate was tested showed susceptibility to amphotericin B, but resistance to both itraconazole and fluconazole[10]. In a pathogenicity study using cane toads, Stewart and Munday (2004)[12] found that the positive mating strain and platypus-derived isolates of *M. amphibiorum* were more pathogenic than negative mating strains or anuran-derived isolates. In a disinfectant trial, a positive mating strain of *M. amphibiorum* from a platypus was more resistant to disinfectants (Phytoclean®, Path-X®, F10sc®) than a negative strain from a frog[13]. *M. amphibiorum* was not isolated from 40 faecal or 8 healthy skin samples from platypuses or 14 environmental samples including soil, water, frog faeces, and *Ixodes ornithorhynchi* ticks. *Mucor circinelloides* was isolated from samples of soil, platypus and frog faeces; *Mucor hiemalis* was cultured from platypus faeces and *Mucor saturninus* from soil samples from the study site. *Mucor circinelloides* was reported from one platypus ulcer[14], but was later thought to be a contaminant as it was incapable of infecting cane toads[12].

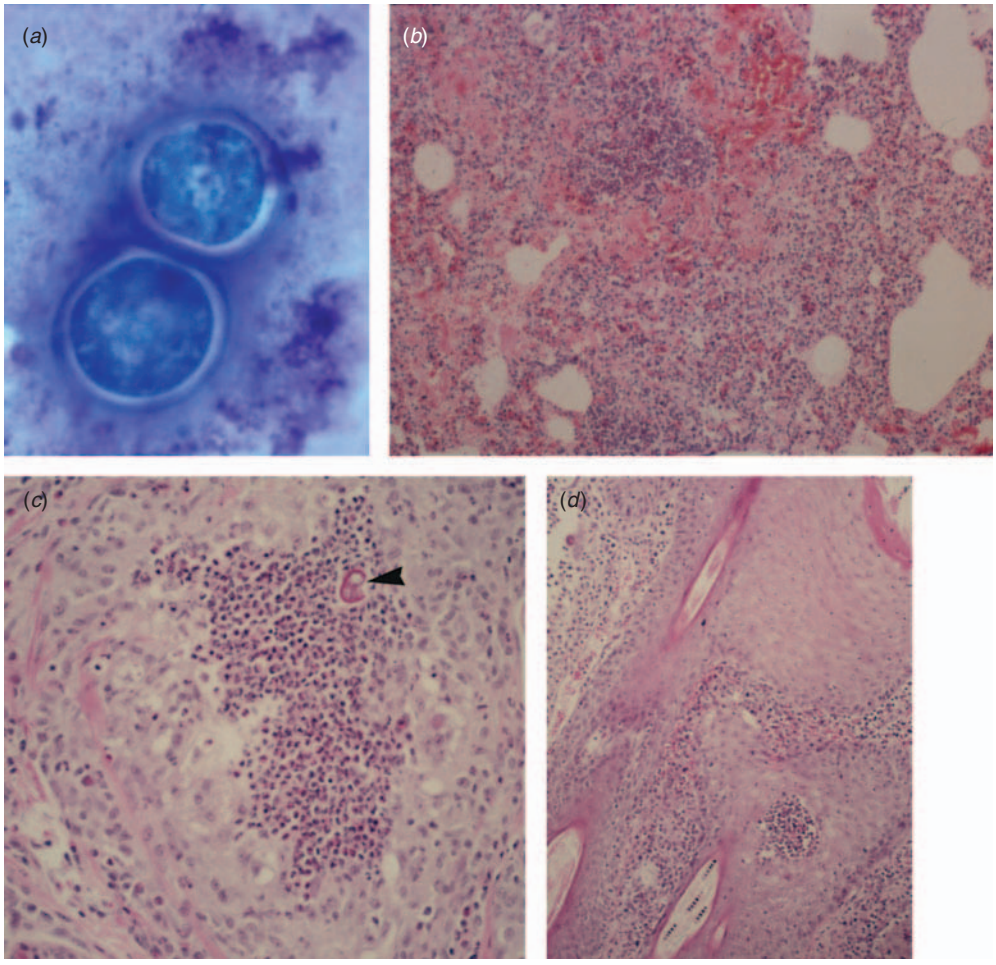

Figure 2. Cytological and histological features of mucormycosis in the platypus. (*a*) *Mucor amphibiorum* in a Diff Quik-stained smear from a case of platypus mucormycosis. (*b*) Granulomatous pneumonia in a platypus lung (H&E, x200). (*c*) Central neutrophils and a ruptured spherule (arrowhead) surrounded by macrophages, lymphocytes and plasma cells in a discrete granuloma (H&E, x280). (*d*) Pseudoepitheliomatous epidermal hyperplasia in a thigh lesion from a platypus (H&E, x140).

The sudden emergence of mucormycosis in Tasmanian platypuses in 1982 may have resulted from accidental introduction of this pathogenic fungus with 'banana box frogs' from Queensland[12] to a naïve Tasmanian platypus population (similar to the recent introduction of the chytrid fungus into Tasmania[15]). Alternately, an endemic Tasmanian strain of *M. amphibiorum* may have mutated to become pathogenic for platypuses[16,18]. Since the index cases of mucormycosis in the platypus in 1982[8], the distribution of the disease has slowly expanded but remained endemic to the catchments draining into the Tamar River. Spread of the agent could be via movement of platypuses and other aquatic hosts or fomites such as contaminated fishing gear and tyre treads. In 1994, mucormycosis prevalence in the platypus at Brumbys Creek was 33%[10]. By 2009, the prevalence of platypus mucormycosis across Tasmania appeared to be declining[17,18].

Diagnosis of mucormycosis in frogs, toads and the platypus

Diagnosis of mucormycosis is based on culturing *M. amphibiorum* from characteristic lesions. Aseptically collected representative specimens (including fine needle aspirates, swabs and punch biopsies) should be inoculated onto Sabouraud's dextrose agar with and without gentamicin (50 IU/mL) and incubated at 28°C. Single colonies can then subcultured onto plates containing Sabouraud's dextrose agar without antibiotics or potato dextrose agar for more detailed morphological studies and mating experiments[8]. Two mating strains, CBS 763.74 (positive type strain) and CBS 185.77 (negative reference strain) were used to assess zygospore production in aerial hyphae[2]. By definition, positive strains produce zygospores only in test matings with negative strains.

Clinical signs (Figure 1), the presence of spherules in cytology preparations or histological sections from lesions (Figure 2) further support a diagnosis of mucormycosis in anurans or platypus, but are less specific than culture. *Corynebacterium ulcerans* and an unidentified fungus were isolated from cutaneous lesions resembling mucormycosis in two platypuses[18]. Several environmental *Mucor* species other than *M. amphibiorum* display dimorphism including *M. circinelloides*, *M. hiemalis* and *M. saturninus*, and could potentially result in similar-appearing spherules in lesions. In the platypus, *M. amphibiorum*-specific serum immunoglobulin may be detected by ELISA[19]. To date, no PCR has been used to identify *M. amphibiorum* DNA from clinical (platypus and amphibian) or environmental samples, although panfungal PCR

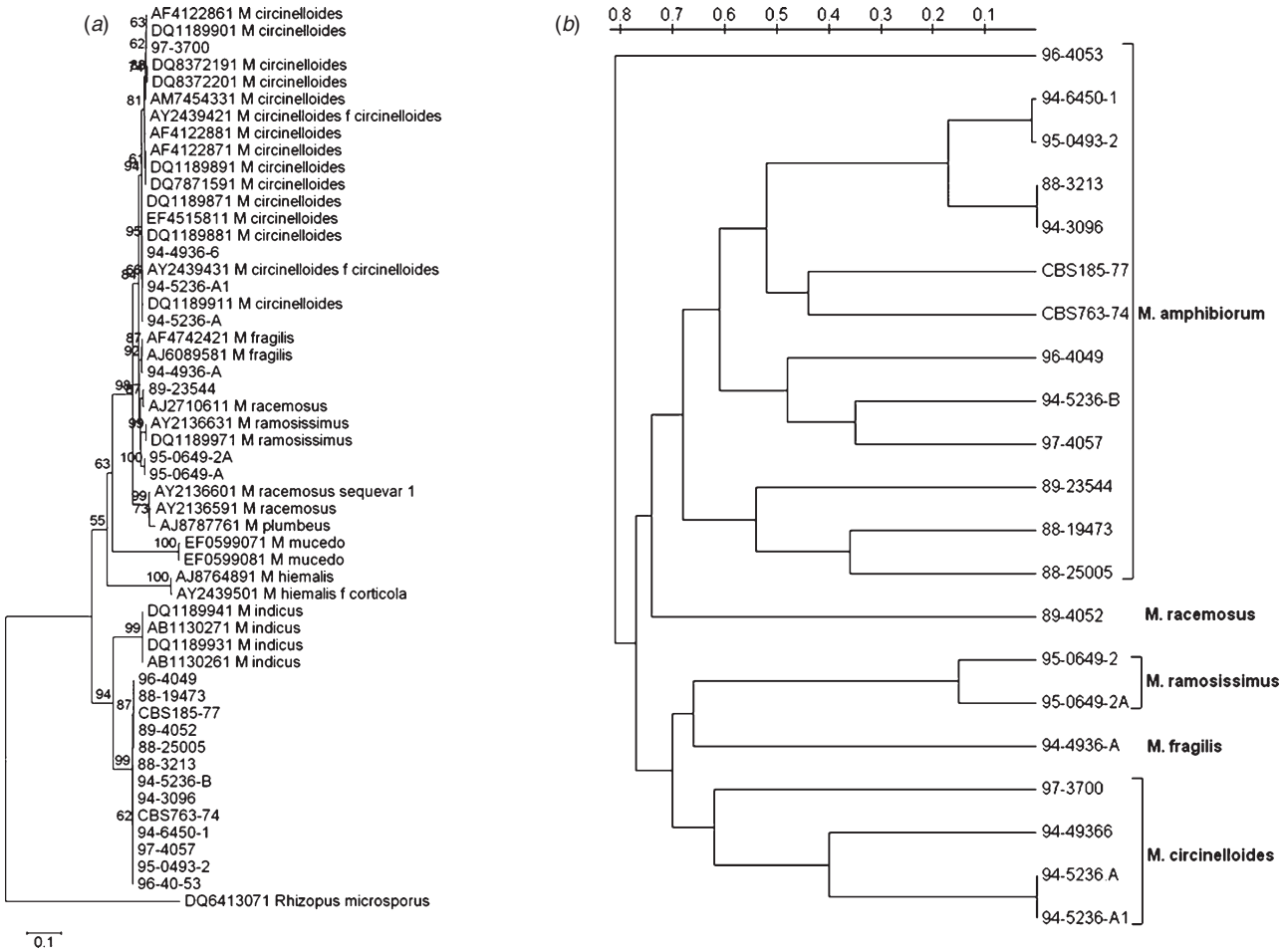

Figure 3. Genotypic analysis of *Mucor* spp. Isolates[20]. (*a*) Consensus Neighbour-joining tree generated from sequence alignments of the rDNA ITS regions of *Mucor* sp. isolated from platypus and species from the GenBank database. Bootstrap support values are indicated for each branch. (*b*) Dendrogram based on the genetic differences as determined by analysis of 135 amplified fragments generated from ISSR amplification.

assays have been used to detect a range of fungi in fresh and paraffin-embedded tissues[20,21] and the fungal microbiome of canine duodenal samples[22].

Molecular studies of *Mucor* spp. of platypus, anuruan and environmental origin

A collection of 21 *Mucor* isolates representing isolates from platypus, frogs, toads and environmental samples were obtained for genotypic analysis (Figure 3)[23]. Internal transcribed spacer (ITS) region sequencing and GenBank comparison confirmed the identity of most isolates. Platypus isolates formed a clade containing the reference isolates of *M. amphibiorum* from the CBS repository. The *M. amphibiorum* isolates showed close sequence identity with *Mucor indicus* and consisted of two haplotypes, differentiated by single nucleotide polymorphisms within ITS1 and ITS2 regions. Except for one, all isolates from platypuses were in one haplotype. Multi-locus fingerprinting via the use of intersimple sequence repeats (ISSR) PCR identified 19 genotypes. Two major clusters were evident: (1) *M. amphibiorum* and *Mucor racemosus*; and (2) *Mucor circinelloides*, *Mucor ramosissimus*, and *Mucor fragilis*. Seven *M. amphibiorum* isolates from platypuses were present in two subclusters, with one isolate appearing genetically distinct from all other isolates. Isolates classified as *M. circinelloides* by sequence analysis formed a separate subcluster, distinct from other *Mucor* spp. The combination of sequencing and multilocus fingerprinting has the potential to provide the tools for rapid identification of *M. amphibiorum*.

Future work should include the development and refinement of molecular tools to detect free-living forms of *M. amphibiorum* in the environment as well as infective forms in tissue lesions. The potential for other aquatic vectors for *M. amphibiorum* needs to be assessed. Such developments will likely lead to an improved understanding of the environmental niche of the fungus and how it is spread in Tasmania. This could lead to control measures to prevent further spread of this disease.

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Biography

Joanne Connolly teaches Veterinary Microbiology and is the Course Coordinator of the Captive Vertebrate Management Program at Charles Sturt University in Wagga Wagga. The major themes of Dr Connolly's research are veterinary microbiology, public health, as well as wildlife biology and disease. Research topics of interest include *Mucor amphibiorum*, *Cryptococcus neoformans*, *Salmonella*, *Campylobacter jejuni*, *Escherichia coli* and *Chlamydophila* in animals and host-agent-environmental relationships.

Rethinking the targets for antifungal development

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***Cryptococcus neoformans* is the leading cause of fungal meningoencephalitis and one of the major causes of death in immunocompromised individuals; this AIDS-defining illness has a reported fatality rate of up to 20% in high-income countries such as Australia, and as high as 65% in developing nations[1,2]. The current treatment regime recommended by the World Health Organization is induction therapy with flucytosine and amphotericin B, followed by maintenance and consolidation therapy of fluconazole[3]. Development of resistance to these drugs is an ever-present threat given the pathogen undergoes microevolution while infecting the host, with evidence that this contributes to the high rate of relapse. It is therefore essential that we develop additional classes of antifungal drugs, particularly ones that are more effective than those currently available. But due to the shared eukaryotic physiology of fungi and humans, gross differences that can be exploited as drug targets such as those targeted by current antifungals are limited.**

Rather than focus on large differences between fungal and human physiology, one approach that can be taken in the pursuit of new antifungal targets is a rational drug design approach to exploit subtle differences in otherwise conserved pathways. Rational drug design was pioneered in the purine metabolic pathway[4], and this is one of the pathways providing exciting new avenues for antifungal development. The purine metabolic pathway is extremely well characterised in humans, and is the target for drugs such as mercaptopurine and mycophenolic acid that compromise cells with an increased demand for nucleotides due to their rapid proliferation[5]. However, little investigation has been undertaken into purine metabolism as a potential antifungal target. Given its environmental niche of purine-rich pigeon guano, *C. neoformans* is an ideal candidate in which to study the necessity of purine biosynthesis during infection.

With the aid of a phosphoribosyltransferase, *C. neoformans* can readily salvage purines from pigeon guano in its environmental niche. However, upon entering the human host, purine availability plummets[6] and the pathogen becomes dependent on *de novo* biosynthesis of ATP and GTP. Several studies have shown the dependence of *C. neoformans, Aspergillus fumigatus* and *Candida albicans* on this primary metabolic pathway during the infection of an animal host, a dependence that could be exploited. This potential is currently being investigated through study of *C. neoformans* IMP dehydrogenase, the first dedicated step in the synthesis of GTP from the purine intermediate inosine monophosphate. *C. neoformans* strains lacking IMP dehydrogenase are avirulent in a murine model of infection, and efforts to develop a fungal-specific IMP dehydrogenase inhibitor are being facilitated by the elucidation of high resolution crystal structures. While sequence alignments reveal high identity of this enzyme between the host and pathogen, comparison of the fungal and animal crystal structures has enabled the identification of a striking conformational change in the active site pocket. With the aid of *in silico* docking studies, this difference is informing the design of what may be a new class of antifungal drugs[7].

While the study of purine metabolism as an antifungal target builds upon a vast repository of information, an alternative approach is to initiate studies of completely uncharacterized fungal pathways that have been shown to be associated with the infection process. One method of identifying these genes is through microevolutionary studies. Upon infection of a mammalian host *C. neoformans* faces new environmental challenges as it moves from its traditional niche into a new hostile environment requiring its rapid adaptation for survival. Infection occurs via inhalation of fungal cells that lodge in the alveoli and disseminate into the bloodstream. From there, the infection is able to cross the blood brain barrier to cause meningoencephalitis. Importantly, even if this infection is cured relapse subsequently occurs in 6–23% of cases[8]. Studies of relapse isolates

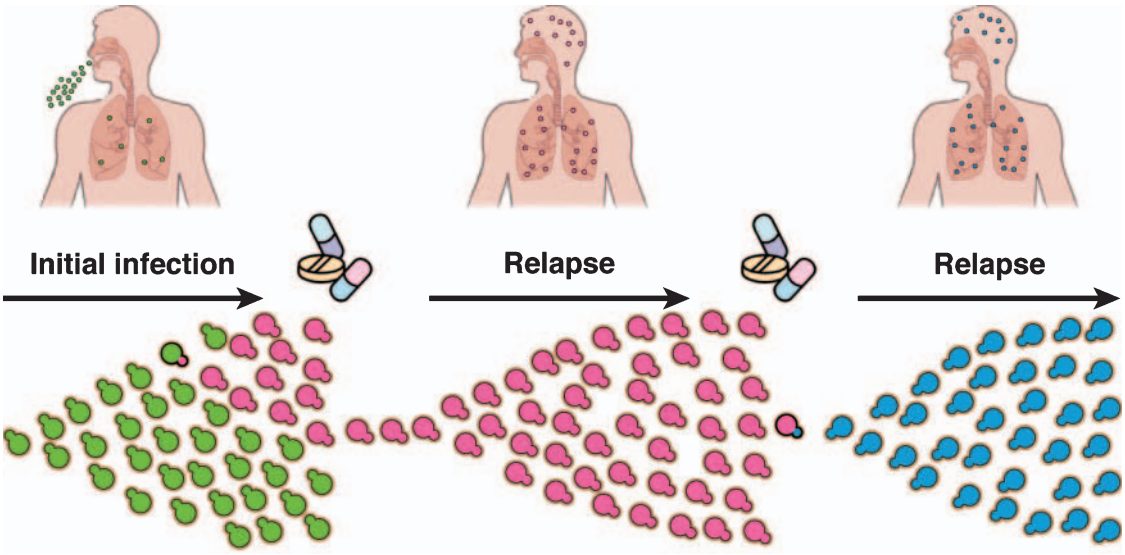

Figure 1. Evolution of *C. neoformans* in the human host. Whole genome analysis has shown that relapse isolates are usually microevolved ancestors of the strain that caused the original infection. How the acquired mutations play a role in pathogenesis, and whether they drive relapse, can assist in design of future drug development efforts.

has revealed that they often exhibit phenotypes that differ from the original infection isolate, and it has been proposed that this microevolution likely facilitates relapse[1,9,10] (Figure 1).

Comparison of the closely related *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* and *Cryptococcus gattii* genomes have shown the genome to be remarkably stable, with no major chromosomal rearrangements in var. *grubii* for the last several million years. However whole genome analyses of clinical isolates from AIDS patients who have suffered from cryptococcal meningoencephalitis and subsequently relapsed have revealed surprising changes. Upon infection of a human host, a microevolutionary burst takes place, with gross chromosomal rearrangements occurring just as commonly as single nucleotide polymorphisms[10]. Furthermore, genome analysis from a range of isolates from several patients has shown a remarkable trend. Of the roughly 7,000 protein coding genes in the genome, several were found to be mutated in independent strains isolated from multiple patients, implying these genes are disadvantageous during the infection process[11]. Unlike the well-understood genes of purine metabolism, these examples of parallel evolution show little homology to characterised genes in other species, making their characterisation more challenging. But excitingly, the information provided by microevolutionary studies is revealing potential vulnerabilities of *C. neoformans* that may help inform the development of much-needed new classes of antimycotic therapeutics.

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Biographies

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Sporotrichosis: an Australian perspective of a global infection



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Sporotrichosis is a fungal infection caused by *Sporothrix schenckii sensu lato* usually acquired after a penetrating injury with contaminated material[1,2]. The infection may establish at the site of the injury, potentially disseminate along the lymphatics, or rarely cause systemic infections including occasional primary pulmonary sporotrichosis[3]. New knowledge of the organism reveals a diverse infection with regard to its epidemiology, geographical distribution, and species characteristics.

S. schenckii sensu lato is a thermally dimorphic fungus characterised by its ability to grow as a yeast-like organism at 35°C (i.e. at body temperatures, Figure 1*a*). However at temperatures <30°C it grows as a mould (Figure 1*b*) demonstrating both clavate/subglobose conidia on denticles arranged in clusters on a short conidiophore (Figure 2*a*), and larger pigmented sessile conidia which may proliferate with age to appear like sleeves along the hyphae (Figure 2*b*).

Despite its dimorphic characteristics, 18S rDNA sequencing indicates *S. schenckii sensu lato* is related to the environmental saprophyte *Ophiostma stenoceras* rather than other clinically relevant dimorphic fungi[1]. In the environment *S. schenckii sensu lato* is mostly associated with a variety of dead organic substrates, with growth accelerated by warmth and humidity. The environmental mycelia produce abundant conidia that may establish areas of endemicity, but when implanted into the body may cause infection in a variety of warm blooded animals. Zoonotic transmission has

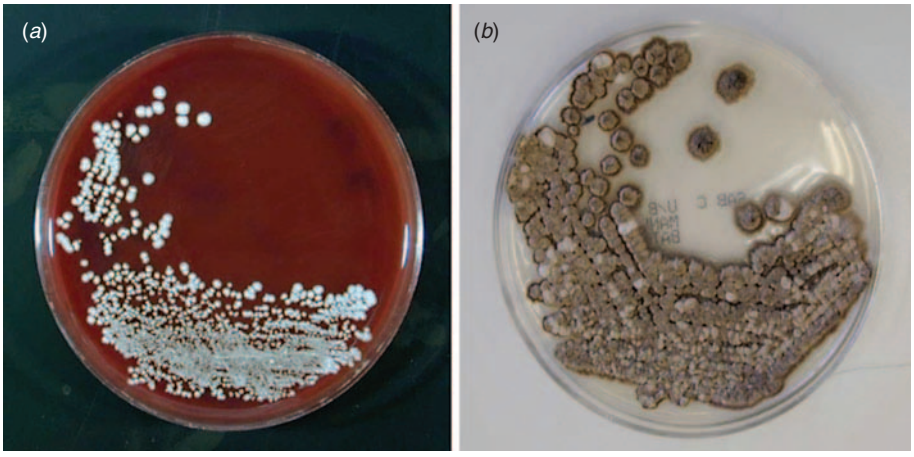

Figure 1. Culture of *S. schenckii sensu stricto* grown on blood agar at 36°C (*a*), and SDA at 26°C (*b*).

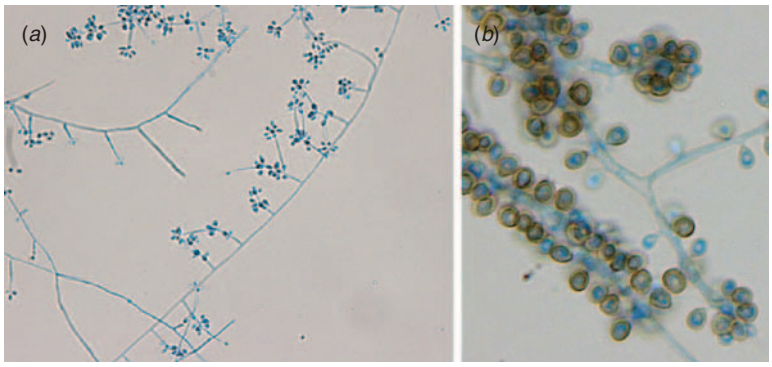

Figure 2. Microscopic features of *S. schenckii sensu stricto* demonstrating the arrangement of conidia on the conidiophore (*a*) and 'sleeves' of pigmented condidia (*b*).

been recorded amongst armadillo hunters and from other animals, but cat-to-cat and cat-human transmission is notable[2].

Patients with sporotrichosis outside of endemic areas may go undiagnosed having undergone unsuccessful treatment with antibacterial agents[2]. Despite the organism growing on a wide range of laboratory media, diagnosis may be hampered by the lack of extended incubation of microbiological cultures or a lack of recognition of the organism particularly if the yeast phase is disregarded as a non-albicans *Candida*.

At least as early as 1979, differences in the virulence and morphology of clinical isolates were noted[4]. Now supported by epidemiological, morphological and molecular data, the species are considered members of a species complex with the main clinically relevant species being *S. schenckii sensu stricto*, *S. brasiliensis*, *S. globosa* and *S. luriei*.

Outbreaks have been reported in geographical localities on all continents other than Europe usually associated with specific organic substrates (e.g. hay/Australia, wood/ South Africa, sphagnum moss/USA, etc.)[1]. Any age/gender bias is predominantly driven by the source of transmission (e.g. male miners in South Africa acquiring the infection from contaminated timbers).

The exception to this global pattern occurs in Brazil where both *S. schenckii sensu stricto* and *S. brasiliensis* have been recorded. *S. schenckii sensu stricto* infection follows the classical sporotrichosis epidemiology across different regions of the country[5]. However, since the early 1990s *S. brasiliensis* has been the agent of an expanding outbreak radiating out from Rio de Janeiro and affecting many thousands of people. Remarkably this outbreak is primarily an urban zoonosis perpetuated by feline sporotrichosis. Cats not only carry the organism but they are also susceptible to infection which is transmitted via scratches from infected animals or from the environment. Given the low level of available medical and veterinary care in these poor socio-economic areas, it seems inevitable the infection will continue to spread across an increasing geographical area affecting many more individuals.

Consistent with epidemiological pattern of these species, it has been demonstrated in a separate report that *S. brasiliensis* is the most virulent of the species in a murine model followed by *S. schenckii sensu stricto* and then *S. globosa*, while *S. mexicana* and *S. albicans* show little or no virulence in this model of infection[6]. Thus expanding knowledge of the different species within the species complex gives understanding of the different epidemiology in different geographical areas, but may also indicate somewhat different susceptibility profiles of the organism[5,7].

Sporotrichosis in Australia

The infection has variously been reported in areas of eastern Australia. In a review of sporotrichosis from NSW, Sivagnanam *et al.* described 31 cases between 2000–2010 around the Port Macquarie area[8]. Sporotrichosis has also been reported from Queensland including a case cluster in 1998 of 16 patients reported in the Darling Downs district of south-east Queensland[9]. In an earlier review 37 cases were described from the Royal Brisbane Hospital between 1965–1977[10].

In Western Australia (WA), sporadic cases have occurred at least since 1975[11], mostly in certain districts of the 'wheat belt' in the south-west. However between 2000-2006 a cluster of 33 laboratory confirmed cases was reported from the Margaret River region where sporotrichosis had not previously been reported[12]. A further cluster of 29 cases were identified from 2011–2014 in the same region. Following investigations of the 2000 outbreak, a local media information campaign was instigated by local health authorities and a 'clean up' of an implicated hay supplier resulted in a reduction in cases. A similar community education programme instituted in 2013 seems to also have resulted in a reduction in the incidence of infection (Figure 3).

Then in 2014 the first cases of sporotrichosis were reported in the Northern Territory[13]. Again, hay was implicated as the infection source, following a remarkably similar pattern to the Margaret River infections.

The species distribution in Australia has only been studied to a limited degree. In a poster presented at the 17th ISHAM Congress in Tokyo from a small sample of isolates, all WA isolates were identified as *S. schenckii sensu stricto*, in comparison to isolates from eastern Australia identified as *S. schenckii sensu stricto* (but a separate clade to those in WA) or *S. globosa*[14].

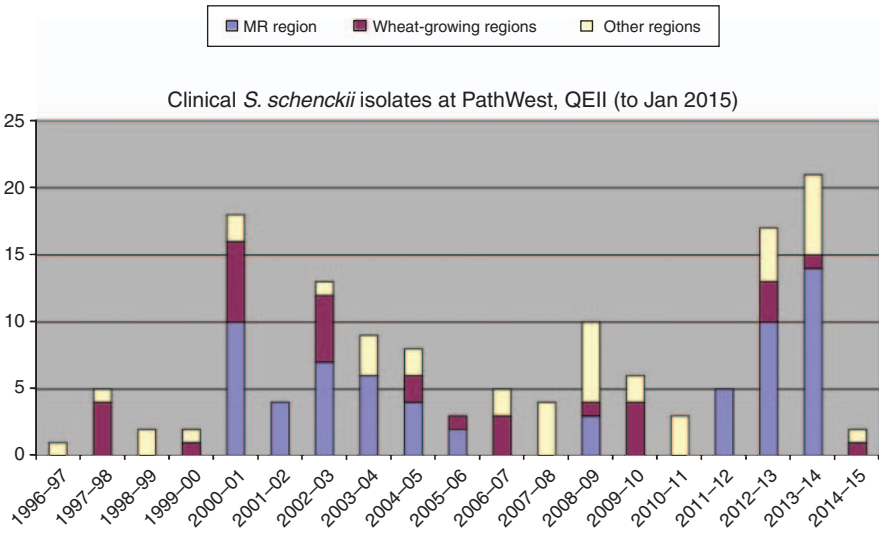

Figure 3. Number of clinical isolates of *S. schenckii sensu lato* recorded at PathWest, QEII, WA, recorded July–June annually to January 2015. MR, Margaret River region.

Conclusion

Sporotrichosis in Australia occurs as sporadic cases in certain geographical regions, with the occasional case cluster often associated with contact with hay. Distribution of organic substrates may spread the organism to new geographical areas. However, the species distribution and susceptibility pattern of the organisms have yet to be systematically studied in Australia.

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Biographies

Ian Arthur is the Senior Scientist of the Mycology Laboratory at PathWest, QEII Medical Centre where he has worked since 1992 with experience in all aspects of clinical diagnostic Mycology. He oversees the operations of the diagnostic laboratory also presenting several university lectures at UWA. The laboratory has had a long interest in superficial mycology, which now uses classical and molecular identification techniques to identify the full range of fungal pathogens. Ian is a former WA ASM branch committee member and treasurer.

Michael Leung is a clinical microbiologist and the regional microbiologist at PathWest Laboratory Medicine, QEII Medical Centre, and also a Clinical Senior Lecturer at the School of Pathology & Laboratory Medicine, University of Western Australia. He was previously Head of Department, Microbiology, at Western Diagnostic Pathology. He obtained his MBBS from the University of Melbourne (1987) and FRCPA (microbiology) in 1997. He has had a longstanding interest in clinical diagnostic mycology.

Inger Elin Westergaard completed her BSc at the University of Bergen, Norway, in 2011, and graduated from Master of Infectious Diseases at UWA in 2014. In her master thesis she investigated the possible source of the sporotrichosis case cluster that occurred in the Busselton-Margaret River region in 2011–2014. She is currently studying for her PhD at UWA, in a co-operation with the Norwegian Public Health Institute. Her research interests include rapid detection of antimicrobial resistance and improving public health in developing countries.

You are what you secrete: extracellular proteins and virulence in *Cryptococcus*




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Fungal organisms secrete a wide range of biomolecules, including degradative enzymes that are essential for nutrition, toxins, effectors and secondary compounds that modulate interactions with host animals and plants, and a variety of signaling and stress-related proteins[1]. As these are likely to be key determinants of virulence and may also be useful diagnostic and therapeutic targets, we investigated the secretome of different strains of the fungal pathogen *Cryptococcus*. Virulent strains secreted predominantly hydrolytic and proteolytic enzymes, while the least virulent strain secreted a range of additional non-degradative proteins including many that lacked secretion signals, some that appear to be 'moonlighting', and a number that are known to be allergenic. It appears that in *Cryptococcus*, the secretome may influence virulence both through the presence of harmful enzymes and through the absence of proteins that alert the host defence mechanisms.

Cryptococcus is an encapsulated yeast with two predominant pathogenic species: *Cryptococcus neoformans* and *Cryptococcus gattii*. These cause cryptococcosis in animals and humans, with disease ranging from asymptomatic to severe, fatal meningitis. There are a number of differences between *C. gattii* and *C. neoformans* including their preferred environmental niche, basidiospore morphology, drug susceptibility, epidemiology, the clinical manifestations of associated disease, and host susceptibility[2]. In addition, there are significant differences among stains within each species. In *C. gattii*, a hypervirulent sub-genotype designated VGIIa has caused a recent significant outbreak of cryptococcosis on Vancouver Island in British Columbia, Canada and in the Pacific Northwest of the United States. In contrast, a closely related sub-genotype designated VGIIb is globally distributed and hypovirulent[3]. These differences between *Cryptococcus* species and sub-genotypes provide an opportunity for understanding pathogenicity and disease progression by what are otherwise very genetically similar fungal organisms.

Our laboratory has been using 'omics approaches to understand virulence in *Cryptococcus*, and used proteomic analysis to characterize the secretome produced by three *Cryptococcus* strains. Two strains were of high virulence (*C. neoformans* and *C. gattii* sub-genotype VGIIa) and the third of low virulence (*C. gattii* sub-genotype VGIIb). In our previous work on *Cryptococcus* proteomics, we found conditions optimized to simulate those encountered in the host induced the production of large amounts of shed capsular material, which interfered with the isolation and identification of proteins[4]. Therefore, we developed a novel method of protein capture using BioRad ProteoMiner™ beads, followed by mass spectroscopy. Sixty-seven cryptococcal proteins were identified and only one was common to all three strains. The secretomes of the high virulence *C. neoformans* and *C. gattii* VGIIa strains were similar and mostly consisted of a hydrolytic and proteolytic proteins. In contrast the lower virulence *C. gattii* VGIIb strain had a larger

number of proteins with a greater diversity of functions (Figure 1). A significant proportion of these proteins are known to have roles in metabolism, signaling/transport, glycolysis and redox processes, and are considered to be canonical intracellular proteins. Published studies have reported very similar proteins in the extracellular milieu of various cell types from other organisms, and there is growing evidence that these may have 'moonlighting' functions, where they participate in completely different processes in alternative environments[5]. An additional subset of proteins found only in the *C. gattii* VGIIb secretome were orthologous to proteins known to elicit an immune response in the host, including the glycolytic proteins enolase and glyceraldehyde-3-phosphate dehydrogenase[6,7]. Most of these unusual secreted proteins lack secretion signals and are likely to be exported via alternative secretion pathways such as inside microvesicles, which have previously been isolated from *Cryptococcus*[8]; indeed the regulatory 14-3-3 protein, which is considered a biomarker of microvesicles[9], was present exclusively in the VGIIb secretome.

Mammals have a high level of innate immunity to most fungi, and the ability to infect immunocompetent hosts remains a rare trait. *Cryptococcus* is an environmental fungus, and as it cannot be spread from host to host, mammalian infection is likely to be accidental[10]. The questions of what determines virulence, and what processes underlie the evolution of strains that cause significant outbreaks in a dead-end host, are therefore intriguing. Comparative genomic studies have identified genes that are particular to the high virulence strains but their role in virulence is yet to be verified[11,12]. As secreted biomolecules are mediators of contact between the host and the pathogen, differences in these are likely to influence whether a pathogen will be rapidly recognized and eliminated, or will be able to bypass the host response and use host resources to establish an active infection. The results of our secretome analysis suggest that virulence in *Cryptococcus* may in part be determined by restricted secretion of proteins likely to elicit an immune response, and that in the absence of these the production and secretion of degradative enzymes enables host invasion.

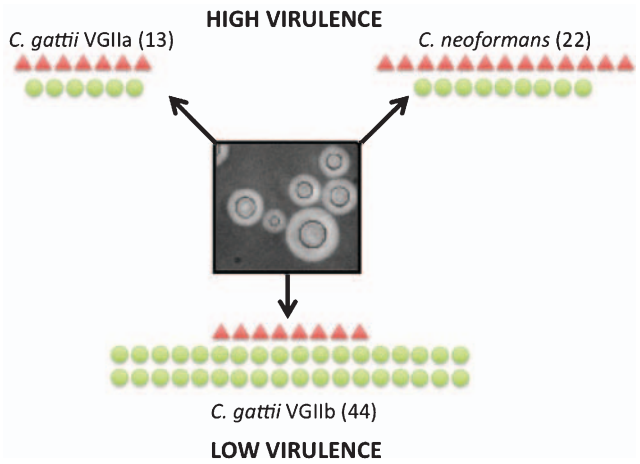

Figure 1. The secretome of high and low virulence strains of *Cryptococcus*. Red triangles: hydrolytic and proteolytic enzymes; Green circles: proteins involved in metabolism, signaling/transport, redox, stress responses or with unknown function.

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Biographies

Leona Campbell has spent the past 13 years being fascinated by the fungal pathogen *Cryptococcus*, both as a PhD student and Postdoctoral researcher, primarily in Dee Carter's lab. Her major area of interest is investigating host-pathogen interactions using 'omics' approaches. Leona also enjoys her teaching role overseeing the running of intermediate undergraduate Microbiology practical courses at the University of Sydney. She loves having the opportunity to inspire, and be inspired by, our next generation of microbiologists.

Dr Matt Padula is a Lecturer in the School of Biological Sciences and Professional Officer in the Proteomics Core Facility at the University of Technology Sydney. His research lies in the proteomic analysis of a range of organisms such as bacteria, yeast, mammalian

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Liz Harry is a Professor of Biology and Deputy Director of the ithree institute (infection, immunology and innovation) at the University of Technology, Sydney (UTS). Liz obtained her PhD at the University of Sydney, was an NIH Fellow at Harvard, an Australian Research Council (ARC) Postdoctoral Fellow and an ARC QEII Fellow in the School of Molecular Biosciences at the University of Sydney. She has won an Australian Eureka Prize for Scientific research, and an ASM Frank Fenner Award. Her research focuses on bacterial cell division and antibacterials.

Dee Carter is an Associate Professor and head of the Discipline of Microbiology in the School of Molecular Bioscience, The University of Sydney, where she teaches mycology, medical microbiology and molecular biology. Her current research interests focus on using 'omics approaches to understand fungal pathogenesis and to develop novel antifungal agents. She loves fungi because they are so adaptable and clever, making them excellent pets but also devastating enemies. She is particularly fond of *Saccharomyces* because it fits into the former category, *Cryptococcus* because it fits into the latter, and *Aspergillus* because it manages to straddle both.

Morphogenesis and pathogenesis: control of cell identity in a dimorphic pathogen

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Fungal pathogens span all major phylogenetic groupings within the fungal kingdom, infecting animals, plants and other fungi. Intrinsic to their ability to infect a host and survive host defense mechanisms is the capacity to produce the appropriate cell type. The link between morphogenesis and pathogenesis is clear for a number of pathogenic fungi that undergo a phase transition known as dimorphism (or dimorphic switching)[1]. Dimorphic fungi are able to alternate between multicellular filamentous growth, characterised by highly polarised hyphal growth, and unicellular growth with yeast cells dividing by budding or fission. This trait is strongly linked with virulence in the important human pathogens *Blastomyces dermatitidis*, *Candida albicans*, *Coccidioides immitis/posadasii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis/luttzii*, *Talaromyces marneffei* (formerly named *Penicillium marneffei*) and *Sporothrix schenckii*[1]. Uncovering the mechanisms that control morphogenesis during dimorphic switching and the physiological properties of the hyphal and yeast cell types is crucial to understanding pathogenicity.

Prevalent in South-East Asia and the surrounding regions, *T. marneffei* causes a deadly systemic infection in immunocompromised hosts[2,3]. The rapid rise in *T. marneffei* infections associated with the worldwide HIV pandemic led to it being described as an AIDS-defining pathogen[3]. While there are sporadic reports of *T. marneffei* infections in 'immunocompetent hosts' the immune status has not been adequately tested in these cases, and the term 'immunocompetent' is often used interchangeably (and incorrectly) in these reports with HIV negative status. The ecological niche of *T. marneffei* is unclear, but there is a strong association with a number of bamboo rat species in endemic areas[3,4]. *T. marneffei* is unique as the only member of the very large *Eurotiales* order that can undergo a dimorphic switch, and the only 'Penicillium' species within this order known to be a pathogen[5,6].

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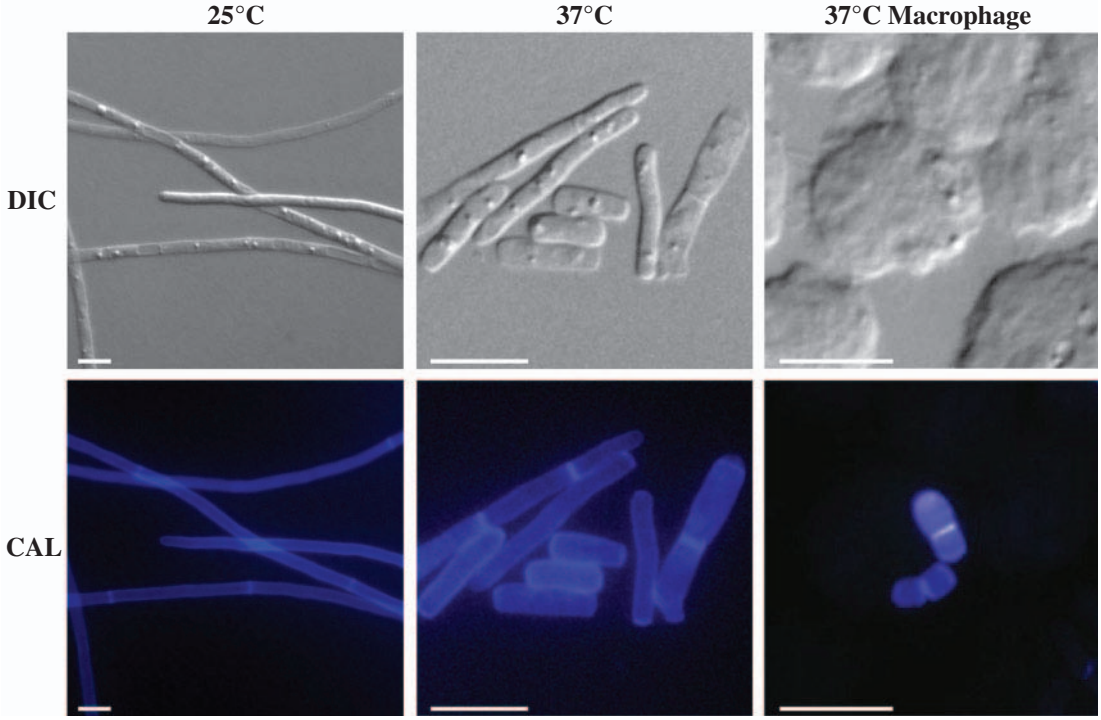

Figure 1. The major cells types associated with dimorphic switching in *T. marneffei*. Microscopic images obtained under differential interference contrast (DIC) or fluorescence microscopy after staining with calcofluor white (CAL) to highlight the fungal cell wall. The hyphal (25°C) and yeast (37°C) cell forms grown *in vitro* for 2 and 5 days, respectively. Also shown is the yeast cell form growing in host macrophages (J774 cells) (37°C Macrophage) 24 hours after infection. Scale bars are 10 µm.

As for many dimorphic pathogens, temperature is a key trigger for the dimorphic transition (Figure 1). At 25°C, *T. marneffei* produces multinucleate filamentous hyphal networks (mycelia) by highly polarised apical growth, subapical cell branching and uncoupled nuclear and cellular division. Specialised differentiated aerial hyphae known as conidiophores generate uninucleate conidia, the most likely infectious agent. At 37°C, uninucleate fission yeast cells, which represent the pathogenic form, are produced via coupling of nuclear and cellular division and complete cell separation at centrally located double septae. In the host the yeast cells of *T. marneffei* reside within phagocytes, predominantly macrophages, subverting the killing activity of these cells and proliferating within them[5].

Genetics studies in *T. marneffei* aimed at dissecting the roles of cell signalling and polarity determinants have identified many highly conserved factors including p21-activated kinases (PAKs) and Ras-superfamily small GTPases (Ras/Rho/Cdc42/Rac)[7,8]. In *T. marneffei*, PAKs are key regulators of the temperature-dependent response. Mutants in *pakA* fail to germinate at 37°C either *in vitro* or in host cells[9]. A second PAK also exists in many fungi and it has been shown in *T. marneffei* that PakB is essential for yeast cell morphogenesis during growth in host cells but not *in vitro*[10]. In addition, loss of *pakB* results in the inappropriate production of yeast cells at 25°C. The GTPases *rasA*, *cflA* (encoding a Cdc42 orthologue) and *cflB* (encoding a Rac orthologue) have both overlapping and unique functions. For example, RasA functions upstream of CflA during germination of conidia, hyphal cell polarised growth and yeast cell morphogenesis, whereas CflB is important for conidiophore morphogenesis and hyphal cell branching. Importantly, CflA is upstream of PakA during the transition from conidia to yeast cells at 37°C highlighting a distinct temperature regulated yeast morphogenesis pathway. In many other fungal pathogens orthologous factors to *rasA*, *cflA*, *cflB* and *pakA* have also been shown to affect morphogenesis (for example, Almeida *et al*.[11]).

More recently, upstream factors, important for sensing temperature and the host cell environment, as well as transcriptional processes triggered to effect morphogenesis, have been characterised. A derivative of prokaryotic two-component systems, the hybrid histidine kinases (HKK) are a major class of sensor systems used by fungi to transmit information from the external environment[12]. Two HKKs of *T. marneffei*, encoded by *drkA* and *slnA*, are required for different aspects of yeast morphogenesis in macrophages: SlnA is important for germination and DrkA for the transition to yeast cells. These HKKs also have additional roles including stress adaptation, asexual development, hyphal morphogenesis and cell wall integrity showing that they are key factors in the ability of the various cell types to respond to the external environment and trigger the correct cellular response. In both *B. dermatitidis* and *H. capsulatum*, the DrkA orthologue is essential for the hyphal to yeast transition and mutants are severely compromised in their virulence[13].

At the other end of the spectrum, very few transcription factors have been identified as major regulators of vegetative cell type morphogenesis as it relates to pathogenicity. The *velvet* family of factors play an important role in *H. capsulatum* yeast cell morphogenesis[14] but this is not conserved in *T. marneffei* and these factors have diverse roles in other fungi. In contrast, the *hgrA* gene, encoding a C_2H_2 zinc finger transcription factor, plays a central role in hyphal cell morphogenesis and its activity must be downregulated in order to generate the pathogenic yeast cell type, either *in vitro* or in macrophages[15]. Loss of HgrA also leads to cell wall defects and increased sensitivity to cell wall, oxidative, but not osmotic stress agents. Based on these studies and those in other fungi, it is clear that the HgrA family of transcription factors are conserved regulators.

Future directions

Despite the efforts of many groups around the world, working on a range of dimorphic fungal pathogens, studies into the mechanisms that control this morphogenetic transition, which is central to pathogenicity, are in their infancy. A handful of key factors have been identified and these are excellent entry points into uncovering the network of genes that regulate this process. With the newly developed high-throughput genomic tools such as ChIP-seq that are now available and established in these various dimorphic pathogens, our understanding of these systems is primed to uncover new and exciting avenues for the control and treatment of these infections.

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Biographies

Hayley Bugeja is a post-doctoral research fellow in the School of BioSciences at The University of Melbourne. Her research focus is to understand how gene expression is regulated during growth, development and the establishment of cellular identity using fungi as model systems. This has been fostered through her research dissecting the molecular mechanisms controlling the dimorphic transition, and its contribution to virulence, in the human pathogen *Talaromyces marneffei*.

Alex Andrianopoulos is a Reader in Genetics in the Genetics, Genomics and Development cluster in the School of BioSciences at The University of Melbourne. His research is focused on understanding the fundamental molecular mechanisms that control cellular morphogenesis and development using a number of model fungi and how these mechanisms underpin virulence and pathogenicity in pathogenic fungi.

Use of *Caenorhabditis elegans* as a non-mammalian model system to study *Candida* virulence



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***Candida albicans* forms part of the normal human commensal flora but has the ability to cause serious, invasive disease in those who are immunosuppressed. One of its key virulence determinants is its ability to transition from a yeast to a filamentous form. This article focuses on the utility of using the worm model, *Caenorhabditis elegans*, to study *Candida* pathogenesis. *C. elegans* provides an *in vivo* infection environment that is ideally suited to study the mechanisms of filamentation and its role in disease. Findings from the *C. elegans-Candida* model appear highly predictive of findings in a mammalian infection model.**

C. albicans is one of the most common human fungal pathogens[1]. It is part of the human commensal flora, which colonizes gastrointestinal, mucocutanous, and genitourinary areas in nearly 80% of healthy individuals[2]. However, when host immune defenses are disrupted, severe invasive disease such as candidaemia can ensue[3,4]. One of the most important virulence factors that contribute to *C. albicans* pathogenesis is its ability to switch, reversibly, between yeast to filamentous (hyphal) forms[3]. The hyphal form of *C. albicans* is thought to be intricately related to its pathogenesis, with some studies showing that it is necessary for tissue destruction and invasion[3,5–7].

C. elegans is a soil-dwelling nematode that has been used in biomedical science for over 30 years; however its use for studying microbial pathogenesis is more recent. *C. elegans* provides an excellent balance between complexity and logistic ease, as well as having significant ethical and financial advantages over mammalian infection models[8–10]. *C. elegans* has a fast generation time: it grows up to 1 mm in length and can produce genetically identical progeny in a 3-day life cycle[11]. The worm has been successfully used to uncover host immunity and virulence of pathogens similar to that implicated in human or other animal models during disease. *C. elegans* is a natural host to various pathogens including *Microbacterium nematophilum*, *Drechmeria coniospora*, *Nematocida parisii*, while it has also been used as model host for many pathogenic fungi including *Cryptococcus neoformans*[12], *Histoplasma capsulatum*[13], and most recently *Penicillium marneffei*[14].

Although it has been challenging to find an appropriate non-mammalian model to study the role of filamentation in the pathogenesis of *C. albicans*, Breger and colleagues developed the *C. elegans* model to perform *in vivo* studies of *C. albicans* pathogenesis and antifungal compounds[15]. Using a standard feeding assay, *C. albicans* were ingested by adult *C. elegans* worms, and then worms were transferred into liquid media. *C. albicans* established a persistent

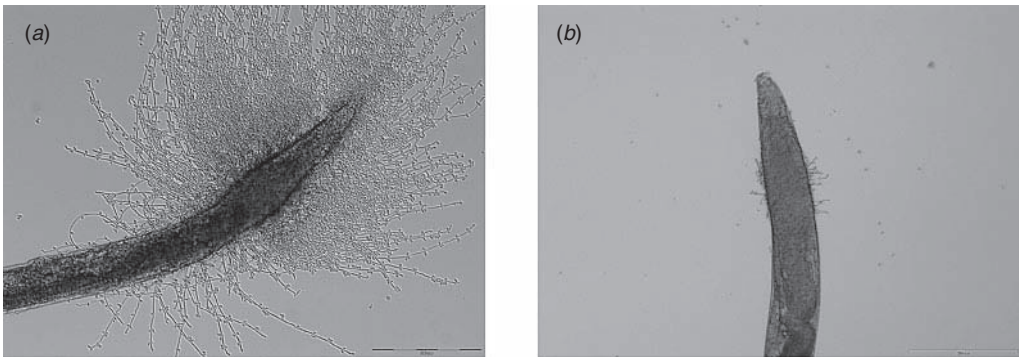

Figure 1. Representative images of a worm infected with wild-type (WT) *C. albicans* (*a*) and a *C. albicans* mutant defective in filamentation (*b*).

intestinal tract infection and proliferated within the gut. The yeast cells would then undergo a morphological transition to form penetrative filaments, piercing the body of the worm and leading to its death. An example of this penetrative filamentation is shown in Figure 1*A*. In some ways, this has similarity to its proliferation in the human gastrointestinal tract and gut translocation to cause invasive disease[15]. Pukkila-Worley, Peleg and colleagues have also shown the successful use of *C. elegans* in elucidating *C. albicans* dimorphism[7]. By employing hyphal defective mutant strains (*EFG1* and *FLO8*) we showed that hyphae formation is necessary for the pathogenesis of *Candida* as confirmed by attenuated virulence toward *C. elegans*. An example image of a worm infected with a hyphal-defective *Candida* mutant is shown in Figure 1*B*. The study further demonstrated the convenience of using the *C. elegans*-*C. albicans* infection model to screen *C. albicans* mutants for genes implicated in virulence. The screening identified the role of two novel genes; *ADA2* and *CAS5* in *Candida* virulence[7]. Interestingly, an *ADA2* deletion mutant showed normal filamentation *in vitro* but showed attenuated filamentation inside *C. elegans*, highlighting the utility of *C. elegans* as a substitute *in vivo* host for characterisation of *C. albicans* virulence[7]. Other *Candida* virulence factors have also been identified using *C. elegans*, including the co-transcription factor known as Mediator Med31 and the mitochondrial outer membrane SAM (Sorting and Assembly Machinery) complex subunit (Sam37), which were both shown to have an impact on *C. albicans* virulence[16,17]. The Sam37 results were also confirmed using a mammalian infection model.

Our group has also extended the use of *C. elegans* to study polymicrobial infections[18]. Our results have revealed interesting interactions between two diverse and clinically important organisms, the bacterium *Acinetobacter baumannii* and the fungus *C. albicans*. We showed that *A. baumannii* inhibits the ability of *Candida* to form filaments in *C. elegans*. This attenuates the virulence of *Candida* as determined by reduced *C. elegans* killing[18].

C. elegans is a powerful model system to study host pathogen interactions and has excellent predictive value for microbial pathogenesis in mammalian models. It is widely used for illustrating mechanisms of virulence in diverse pathogenic organisms including the human fungal pathogen *C. albicans*, enabling the identification of novel mechanisms that would not necessarily be determined using *in vitro* assays.

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Biographies

Farkad Bantun is a PhD student in the Peleg Lab at Monash University, Melbourne, and is working on the genetic mechanisms driving the morphological transition of *Candida* from yeast to a filamentous form using *Caenorhabditis elegans* as an *in vivo* model. He finished his schooling in Saudi Arabia where he also completed his bachelor degree in Medical Technology. He was awarded a scholarship to complete his postgraduate studies at Monash University. He also finished a Masters degree in Laboratory Medicine at RMIT University, Melbourne.

Sanjiveeni Dhamgaye is a postdoctoral research fellow in the Peleg lab working on polymicrobial biofilms and *Candida* pathogenesis. She was born in New Delhi, India, and after her schooling, pursued a career in Science. She completed her PhD at Jawaharlal Nehru University, New Delhi, India, with a focus on 'Regulation of Multidrug resistance in yeast' with Prof. Rajendra Prasad.

Professor Anton Y Peleg is the Director of the Department of Infectious Diseases at the Alfred Hospital and Monash University, and is a research group leader in the Department of Microbiology, Monash University. His research spans clinical to basic research, with a focus on hospital-acquired infections, antimicrobial resistance, infections in immunocompromised hosts and understanding mechanisms of disease caused by hospital pathogens, including *Candida*, *Staphylococcus aureus* and *Acinetobacter baumannii*. His group has expertise in the use of non-mammalian model systems to study microbial pathogenesis.

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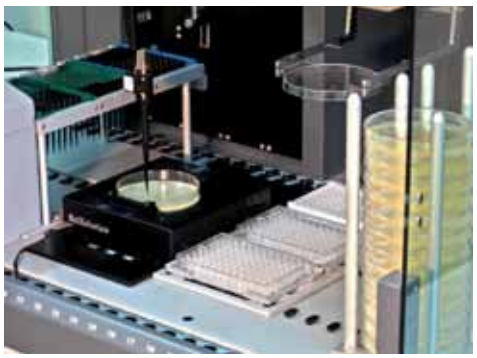

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