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Rising to the challenge of multiple *Cryptococcus* species and the diseases they cause

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Abstract

Cryptococcus neoformans and *C. gattii* are well-studied basidiomyceteous yeasts that are capable of causing disease in healthy and immunocompromised people. The Conference on *Cryptococcus* and Cryptococcosis (ICCC) is held every three years: the accompanying Special Issue stems from the 9th ICCC and covers a subset of the topics related to these fungi in detail. This conference started with a revised and reduced estimate of disease burden globally, in part due to improved treatment for HIV+ people. However, mortality from cryptococcosis remains consistently high for those unfortunate to have limited access to therapies or without underlying immunodefiencies. As such, there are yet still great distances to be covered to address antifungal drug availability, the need for new antifungal agents and the timing and doses of these agents in conjunction with antiviral therapy, underscoring the importance of continued research. A notable point from the 9th ICCC was the research addressing the variation in the pathogen and host populations. Analysis of cryptococcal strain variability, particularly at the molecular level, has resolved distinct lineages with the consequence of a taxonomic revision that divides *C. neoformans* and *C. gattii* into seven *Cryptococcus* species. Similarly, analysis of host factors in so called “immune-competent” individuals revealed previously unrecognized risk factors. Research on these species has established them as important model organisms to understand gene evolution and function in other fungi and eukaryotes. The stage is set for the refinement of research directions, leading ultimately to better treatment of this pathogenic monophyletic clade in the genus *Cryptococcus*.

Keywords

Cryptococcus; *Filobasidiella*; medical mycology; taxonomy

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1. Introduction

Cryptococcus neoformans was first described over a century ago as a yeast growing from an environmental source and from a human patient (Perfect and Casadevall, 2011; Srikanta et al., 2014). Since that time and particularly as a consequence of AIDS, the fairly rare fungus in clinical settings emerged to become one of the world's most problematic killers (Park et al., 2009). The medical mycology community stepped up to face this challenge at the start of the AIDS pandemic, brought in a number of new investigators and explored different angles of the fungus and disease it causes. One consequence was the organization of the International Conference on *Cryptococcus* and Cryptococcosis (hereafter ICCC), held roughly every three years since 1989 (Kwon-Chung et al., 2012). First held in Jerusalem and then subsequently Milan, Paris, London, Adelaide, Boston, Nagasaki, and Charleston, the 9th conference was held in Amsterdam, 15–19 May 2014, bringing together the largest group thus far of 303 investigators to discuss the latest discoveries on the fungus and disease.

In addition to the trends of change and adoption of new technologies that are often presented at scientific meetings, a notable feature of the 9th ICCC was the research indicating that this is neither a single pathogen nor a single patient population. The variability between pathogens and hosts has been appreciated for many years. From the fungal side this has resulted in the separation of strains into different serotypes, varieties, groups based on molecular markers, and establishing two species *C. neoformans* and *C. gattii* (Kwon-Chung, 1976; Kwon-Chung et al., 2002). Hosts include diverse animal species (Malik et al., 2011), and for the human host a separation into immune status such as HIV+, otherwise immunosuppressed, and other unknown susceptibilities. However, there is now greater realization of the different fungal subtypes and their biology and the underlying immune status of the patient population, and consequences in clinical outcome.

This issue has 10 articles that provide the current state-of-the-art in specific areas related to the biology of the *Cryptococcus* species and host factors that shape the outcome of the infection. These are selected to highlight some key areas in the field, and by no means would this collection be considered comprehensive. Two books on the topic are valuable references (Casadevall and Perfect, 1998; Heitman et al., 2011). The abstracts from the talks and posters from the 9th ICCC are also available in a special issue of *Mycoses* (2014 volume 57). We can anticipate a series of exciting publications based on the data presented at the conference. With the rest of this editorial we aim to unite the papers in this issue in a wider context of the latest developments in research on *Cryptococcus* and beyond, and draw attention to the overlaps between particular topics.

2. Cryptococcosis as a major global health problem

How serious a problem is cryptococcosis? A much cited article on the global incidence and impact of cryptococcosis estimated 624,700 deaths each year, within wide confidence intervals of 125,000 to 1,124,900 (Park et al., 2009). Most articles use the 625,000 figure, and the cryptococcosis calculations are the basis of global estimates of mortality due to all fungal diseases [e.g. (Armstrong-James et al., 2014; Brown et al., 2012)]. At the 9th ICCC David Boulware presented revised estimates of cryptococcosis mortality that drop that of

Park et al. by about half. While this is still within the original range proposed by Park et al., this decreased mortality is also in part due to improved treatment that occurred in the last five years and reflects genuine medical advances in some countries. Other talks addressed the changing demographics of the patient population with cryptococcosis. For example, the trend presented by John Perfect at the ICCC was for decreased mortality in the HIV positive population in developed countries, but not amongst the "otherwise healthy" group. This is borne out by recent estimates of mortality rates in the United States, with 80% of people succumbing to the disease in the last 11 years not being HIV positive, in contrast to the early days of the AIDS pandemic (Barragan et al., 2014).

While countries like the U.S.A. and in Europe may have made major inroads in treating cryptococcosis and mortality overall has decreased, other parts of the world are not so fortunate. Dr. Boulware described experiences in testing the timing of the initiation of antiviral treatment in cryptococcosis patients in Uganda (Boulware et al., 2014a), and commented that a medical innovation was a set of hooks placed in the walls from which to hang drips. His comments about the severity of cryptococcosis in Uganda were also supported by Thomas Harrison in the opening address, noting that mortality rates are consistently at about 75% in South Africa. This is borne out by other experiences in Africa. For example, recent studies looking at two doses of fluconazole treatment in Malawi reported 10-week mortality rates at 55 and 57% (Gaskell et al., 2014; Rothe et al., 2013).

One article in this issue covers the raising concerns of cryptococcosis in China (Fang et al., 2015). The impact on cryptococcosis on the world's most populous country remained little known for many years (Yuchong et al., 2012), and the current article provides a snap shot of the progression of this disease and the causative agents. Many of the patients are described as immune-competent, indicating that there may be some genetic or other risk factors associated with this ethnic group. One risk correlates with alleles of the Fcγ receptor that bind opsonized pathogens (Hu et al., 2012). Other articles and talks at the 9th ICCC addressed the distribution of the disease and strains in other parts of the world. In light of the estimates of global disease burden, accurate assessments are vital to provide a rationale to agencies to invest in efforts to combat this disease.

Research on *Cryptococcus* species and the diseases should remain a priority in terms of finding new or refining current strategies for treatment and to elucidate the underlying basic biology needed to bring these to fruition. However, clinical findings provide evidence that treating cryptococcosis is complex, the outcome is heavily influenced by socioeconomic conditions, and that combating the disease requires more than just administration of antifungals to a patient.

3. The *Cryptococcus* species and their detection

A mycology trend has been the revision of the names of species, usually based on insight gained from molecular approaches of cryptic species, but also driven by abolishing sections of the Code of Botanical Nomenclature under which fungi were named (de Hoog et al., 2013). Characterization of *Cryptococcus* strains from around the world has resolved distinct groups, and hence *C. neoformans* and *C. gattii* are further divided into serotypes, varieties,

VN or VG subgroups, or molecular marker profile numbers. It is striking to note that the first talk of the inaugural ICCC in 1989 was titled “Genetic basis for the current taxonomic system of *Cryptococcus neoformans*” as given by June Kwon-Chung, and discussions on revisions to naming – possibly with groups being species – have been spirited at subsequent meetings (Kwon-Chung et al., 2012). Hagen and colleagues provide a new and long-awaited nomenclature for the organisms in what is sometimes termed the “*Cryptococcus neoformans* species complex” (Hagen et al., 2015). There have been challenges to produce a new naming system; for instance, many isolates in different lineages are capable of fusion with others during the sexual cycle. Indeed the type strain for *C. neoformans* is a hybrid of two species (Hagen et al., 2015; Kwon-Chung and Varma, 2006). Under the new nomenclature, *C. neoformans* refers to those strains considered serotype A or var. *grubii*, and *C. deneoformans* for serotype D or var. *neoformans*. Strains of *C. gattii* will be divided into five species: *C. gattii*, *C. bacillisporus*, *C. deuterogattii*, *C. tetragattii* and *C. decagattii*. This nomenclature provides a way to refer to these organisms rather than the current mix of non-standard terms.

An essential step in the treatment of cryptococcosis is first being able to make an accurate diagnosis and to do so as quickly as possible (Perfect and Bicanic, 2015). The methods to do this have changed over time, with the latest being a lateral flow assay (LFA) that relies on antibody detection of the fungal glucuronoxylmannan in the capsule. This assay offers a number of benefits over previous methods, including accuracy (Boulware et al., 2014b; Tang et al., 2015). One disadvantage of LFA is the inability to resolve the strain subtypes (or species) with the pathogenic *Cryptococcus* species clade, as was possible using the serotyping system from Iatron Laboratories (Tokyo, Japan) that is no longer available. It is worth reflecting that the need for rapid and accurate strain classification in different groups was instrumental in the development of molecular-based methods that then revealed underlying unique subgroups within the species [e.g. (Meyer et al., 1999; Meyer et al., 1993)]. At the same time, finding standard, easy to use and cheap clinical tools to define to species level continues to remain a goal.

4. Pathogenicity and disease

The seven species within the *C. neoformans* species complex are a rarity amongst those species in the genus *Cryptococcus* by being pathogenic to humans. MycoBank recognizes 322 legitimate species names in the genus (accessed 29 November, 2014). This monophyletic group within the genus therefore has specific capabilities for virulence. The best-studied factors in *Cryptococcus* linked to causing disease are growth at 37°C, biosynthesis of the pigment melanin, and the production of a polysaccharide capsule. However, with the development of methods for targeted gene replacement and random insertional mutagenesis, genes controlling these three traits and others for virulence are being identified rapidly. The latest developments in identifying new, and in characterizing the classical virulence determinants, were presented during the meeting, and reviewed in this issue by Andrew Alspaugh (Alspaugh, 2015). Dr. Alspaugh described some of his own research at the 9th ICCC on one such virulence factor, the Rim101 transcription factor. Rim101 is involved in the pH sensing and response pathways, and is one of the few transcription factors that have been analyzed widely in medically-relevant fungi. A curious

aspect about the *rim101* mutant strain compared to the wild type is that when it is used to inoculate mice, the animals succumb to disease more or equally rapidly. Yet they do so through differing mechanisms (O'Meara et al., 2013). The *rim101* mutant has an altered cell surface, triggering a different set of cytokines to be induced compared to wild type and the enhanced but damaging pro-inflammatory responses. This finding provides an exciting example of the importance of balanced host response to *Cryptococcus* in order to clear the infection without causing inflammatory tissue damage. It also points to how pathogen variation could relate to host susceptibility differences to influence disease outcome, and underscores the need to consider both the host and pathogen simultaneously in designed experiments. In this issue, there are updates on the molecules that act at the interface of *Cryptococcus* and the host that determine disease susceptibility.

The *Cryptococcus* spp. are described as yeasts but this is just one of the many cell types. In addition to the yeast morphology that is most commonly observed, other cell forms exist that include hyphae (some with fused or unfused clamp connections characteristic of basidiomycete dikaryon hyphae), pseudohyphae, blastospores, basidiospores and titan cells (Wang and Lin, 2012). Cells can have haploid, diploid, higher ploidies, or aneuploid genome compositions. Added to the complexity is the cellular age, which affects the size of cells and their physiology. Each of these cell types likely has different properties, and multiple talks at the 9th ICCC covered these aspects, including by Christina Hull (basidiospores), Kirsten Nielsen (titan cells), Bettina Fries (old cells), and Xiaorong Lin (filamentous cells). However, a number of questions remain to be addressed about these cell types. How relevant are each of these forms in clinical situations and in the environment? What factors control how they form? And, is it possible to translate such information into clinical use, such as through vaccine or targeted drug development? In this issue two reviews cover a subset of the different cell types. Bouklas and Fries review the role of ageing cells in the population (Bouklas and Fries, 2015). Fu et al. provide a perspective of the role of mating between strains of the same mating partner (Fu et al., 2015). Mating generates a suite of different cell types and meiosis is part of an alternation of different cell ploidy. In addition, mating is a source of aneuploid strains (Ni et al., 2013).

The ability to change physiology and the time frames to do so by the fungus are of interest with respect to host interactions. What exactly the fungus encounters and responds to at the initial interaction and during the course of disease is still largely a mystery. Analysis of fungi taken from hosts at different times and tissues provides some clues that the host is an environment that selects for, and potentially drives, cell plasticity. There is evidence that the host selects for older cells (Bouklas et al., 2013), triggers DNA mutations (Magditch et al., 2012), induces the formation of the larger titan cell form in lungs [see review by (Zaragoza and Nielsen, 2013)], and changes to oval shape for brain invasion (Shi et al., 2010). Furthermore, exposure to azole antifungals triggers changes in chromosome numbers that lead to heteroresistance (Sionov et al., 2013; Sionov et al., 2010).

5. Host responses to *Cryptococcus* and host treatment options and limitations

The other side to cryptococcosis is the patient population. Exposure to *Cryptococcus* can be high, as demonstrated by the presence of anti-cryptococcal antibodies from an early age (Goldman et al., 2001), yet not everyone develops cryptococcosis disease. Talks at the 9th ICCC addressed new discoveries about the underlying risk factors in contracting disease in the human population, such as the recent finding of patient populations susceptible to *C. neoformans* and *C. gattii* having autoantibodies to granulocyte-monocyte stimulating factor (Rosen et al., 2013; Saijo et al., 2014), and the correlation of specific Fcγ receptor alleles with susceptibility (Hu et al., 2012; Rohatgi et al., 2013). Two articles in this special issue focus on the host side of the disease. One describes the mammalian cells and their responses to infection (Gibson and Johnston, 2015). The other covers the molecules produced by both the host and fungus and how these impact the outcome of the interaction (Wozniak et al., 2015). *Cryptococcus* spp. have a broad host range, in that many animals other than humans are also affected naturally (Malik et al., 2011). This provides research advantages in terms of using these as models for the disease. Hence, many immunological and virulence studies have used these animal models. However, an absence from the 9th ICCC, in contrast to previous conferences, was presentations from veterinarians. A talk by Mitra Shourian on genetic mapping of susceptibility genes in mice highlighted the potential that these alternative hosts may offer, beyond as substitutes for a human host for virulence assays, for identifying host factors that contribute to disease severity or protection.

There is increasing overlap between research on fungal biology and host immunology. One example is the work in developing a cryptococcal vaccine, with several approaches being used. Different cell forms have been tested, including the pseudohyphal RAM pathway mutants (Fromtling et al., 1979), hyphal strains due to Znf2 overexpression (talk by Xiaorong Lin), and a mutant strain that produces chitosan and not chitin (talk by Jennifer Lodge). Each of these approaches reflects the ability of these altered strains to present different materials enabling host recognition while being attenuated for virulence. Other approaches have included the option to alter the host immune system at the same time, such as using a cryptococcal strain that produces host γ-interferon, a molecule that induces protective effects against *Cryptococcus* (Jarvis et al., 2012; Wormley et al., 2007). Yet another approach, described by Françoise Dromer, is the use of specific protein antigens, such as an aspartyl peptidase, to trigger immunity. Analysis of the effects of these stimuli to the host will help our understanding of cellular and/or humoral basis for protection from subsequent exposure to the fungus.

Cryptococcosis would be an insignificant disease if effective antifungal therapies were available to all patients. The estimates of deaths due to these diseases in the hundreds of thousands each year indicate that this is clearly not the case. There are multiple challenges to effective antifungal treatment, and they include diagnosis, timing of treatment, cost of treatment, efficacy of the drugs, and availability of drugs. A recent discovery is that 89% of South African patients with a relapse of cryptococcosis are reinfected by the original strain for a second time (Van Wyk et al., 2014), raising concerns for gains in drug resistance

(temporarily through heteroresistance or permanently through genetic mutations) or inefficiency of current drug regimens to clear the fungus. This problem extends far beyond *Cryptococcus* spp. as it reflects the overall limited arsenal of antifungals available in medical mycology and in agriculture (Denning and Bromley, 2015; Roemer and Krysan, 2014). Effective antifungal drug treatments are further exasperated by global access: for instance, the parts of the world with highest cryptococcosis burden do not have available either the liposomal formulation of amphotericin B or 5-flucytosine (Loyse et al., 2013). Damian Krysan discusses approaches to speed the discovery of new drugs to treat cryptococcosis (Krysan, 2015). One approach is to take drugs already approved for other purposes and test them for their effect on pathogenic fungi. The approach has already produced results. Examples of "repurposed" antifungal agents active against *Cryptococcus* include anticancer agents (Butts et al., 2014) and the anti-depressant drug sertraline (Zoloft) (Nayak and Xu, 2010; Zhai et al., 2012) that is currently in a phase 3 clinical trial.

6. New techniques and resources for research on *Cryptococcus*

While the initial research on *Cryptococcus* focused on medical aspects, the species complex has emerged as a powerful model set of species for understanding gene functions, eukaryotic biology, and evolution more generally. Some more recent examples include the role of this species in understanding sexual reproduction [e.g. reviewed by (Fu et al., 2015)], the functions and evolution of RNAi and as part of genome defense (Dumesic et al., 2013; Wang et al., 2010; Wang et al., 2012), and how introns impact gene expression (Dumesic et al., 2013; Goebels et al., 2013). One study found little correlation between the sensitivity to chemicals and stresses of mutants made in homologous genes of *C. neoformans* and *Saccharomyces cerevisiae* (Brown et al., 2014), providing experimental evidence that supports the convergent evolution of the yeast form of growth in fungi (Nagy et al., 2014). The *Cryptococcus* species fill a vacuum to be able to understanding fungal and more broadly eukaryotic biology.

Progress on both the fundamental and the applied research on *Cryptococcus* species has been driven by technology (Zhang et al., 2015). Advances have included improvements in gene disruption or silencing, microscopy, genome-wide expression analysis and new animal models. The ease of crossing the *C. neoformans* and *C. gattii* species is an advantage compared to other medical fungi where classical genetics has remained difficult or impossible to achieve under laboratory conditions. Congenic pairs are available for *C. neoformans*, *C. deneoformans* and *C. deuterogattii* that have available genome sequences (Kwon-Chung et al., 1992; Nielsen et al., 2003; Nielsen et al., 2005; Zhai et al., 2013; Zhu et al., 2013). Crosses between non-congenic isolates can enable gene identification through the process of map-based cloning (Lin et al., 2006; Toh-e et al., 2015). In the genomics workshop at the end of the ICCC, Hiten Madhani provided an update on the progress and challenges to make a complete deletion set of the non-essential genes in *C. neoformans*, following similar approaches his laboratory developed for the creation of 1200 gene replacement mutants (Liu et al., 2008). In 2015 he has also made more than 2000 new strains available from the Fungal Genetics Stock Center (www.fgsc.net). The gene deletion resource will be the first for a medically-relevant fungus and the Basidiomycota.

Genomics has increasingly impacted *Cryptococcus* research especially in understanding differences between strains: from the ability to design approaches for multi-locus sequence typing or molecular markers to genomics. Microbiology is at a point where genome sequences of strains are replacing molecular markers. Genome sequences can provide the complete information on gene content, and have many advantages in experimental approaches to test gene functions. For instance, these sequences can reveal potential redundancy in gene functions, and as an example the need to mutate multiple genes before observing a phenotype (Kretschmer et al., 2014).

2014 saw the completion of the first *C. neoformans* (var. *grubii*) genome, one of the best assembled and annotated fungal genomes because of the use of RNA-seq data to confirm gene positions (Janbon et al., 2014). One feature of the *Cryptococcus* species and many other basidiomycetes is their intron-richness, and the role of these introns are under investigation in terms of stabilization of gene expression (Goebels et al., 2013) and in the regulation of transposon expression through a spliceosome stalling mechanism (Dumesic et al., 2013). For the *Cryptococcus* species, five genomes were competed using the Sanger chemistry and whole genome shotgun cloning approaches (D'Souza et al., 2011; Janbon et al., 2014; Loftus et al., 2005; Ni et al., 2013), and now >350 strains have been sequenced using next-generation techniques based on talks in the genomics workshop at the 9th ICCC. Genomic research highlights relate to discovering the origin of the *C. gattii* outbreak in western Canada and the northwest of the United States. The level of resolution gained through genome sequencing of the strains provides evidence for not a single outbreak, instead that several independent starting populations have increased in abundance (Billmyre et al., 2014; Engelthaler et al., 2014; Hagen et al., 2013).

7. Future directions before Rio 2017

The consequences of refining the "*Cryptococcus neoformans* species complex" into seven species and a greater appreciation that the disease affects a wide range of individuals in terms of their immunological status may take time to be realized. However, direct comparisons between the fungal species have already started. One example is the analysis of strains from six of the seven species for their responses to the candidate antifungal agent mycophenolic acid, where the authors found that five were sensitive and only *C. tetragattii* (*C. gattii* VGIV) was resistant (Morrow et al., 2012). A second example, based on genome sequencing data, is the discovery that the RNAi machinery is missing in *C. deuterogattii* (*C. gattii* VGII) (D'Souza et al., 2011).

The major question arising from the research presented at the ICCC is how to prioritize future research efforts. First, which isolates should be the reference ones from each of the seven *Cryptococcus* species for subsequent detailed research or new genome sequencing projects? Should there be more than one wild type strain for each species, such as one of each mating type and ideally a congenic pair of robust mating partners? On reaching a consensus, these strains need to be available for distribution to researchers around the world in a coordinated manner. This is important since microevolution occurs under laboratory conditions, leading to confounding differences between observations of the "same" strain from independent groups (Franzot et al., 1998; Janbon et al., 2014). Hence, these strains

would preferably be freely available from a single common source. Second, there is the need for a way of dealing with the explosion of genomics data and other large data sets to make this more easily assessable. Different questions are likely to be asked by different people, and providing an easy way of studying the genomic, transcriptomic or large scale clinical data and comparing between them would open new directions. Third, within the context of the impetus to study *Cryptococcus* spp., putting the host and pathogen – or now hosts and pathogens – pieces together and implementing this knowledge towards personalized medical care represents a challenge. For instance, the 9th ICCC featured discussion about how even the treatment regimen recommendations for *C. neoformans* vs. *C. gattii* infections are currently the same (Perfect et al., 2010). It could be that one-treatment-fits-all will be the most effective; however, the research covered during the ICCC and as presented in this issue suggests otherwise.

In addition to the need to perform experiments and generate data, another key aspect of tackling a disease is to build a community of clinicians, epidemiologists, and bench researchers. In the opening address, Teun Boukhart noted that three people had attended all nine ICCCs since 1989: June Kwon-Chung, John Perfect, and Itzhack Polacheck. Each and many other regular attendees involved in the research for decades made pioneering discoveries on *Cryptococcus* and medical mycology, and beyond that have been instrumental in sharing their expertise and resources that established a legacy for openness in communication and exchange of research material. The work in recruitment, training, and advocacy for research on cryptococcosis and other mycoses was key for work on these fungi that continues to reveal new fundamental aspects about eukaryotic biology, the evolution of fungi, and in combating these diseases. The 10th conference will be in Rio de Janeiro, Brazil, in 2017. Based on past progress, the three years between ICCC meetings will be an equally exciting time for research on the *Cryptococcus* species and cryptococcosis diseases.

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Highlights

- The 9th International Conference on *Cryptococcus* and Cryptococcosis was in May 2014
- The editorial covers the articles found in this special issue dedicated to *Cryptococcus*
- A revised taxonomy supports seven pathogenic *Cryptococcus* species
- Cryptococcosis is still a global health issue
- Research on *Cryptococcus* spp. has established them as models for fungal biology