A Model CNS Fungal Infection: Cryptococcal Meningitis

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Abstract *Cryptococcus* spp. are considered ‘model yeasts’, and the reasons for that are the topic of this review. Several perspectives will be discussed, starting with their characterization and emergence as human pathogens before moving onto their adaptations and the scientific response. The section on characterization illustrates the progress of knowledge and technology over the 120 years since *Cryptococcus* was first identified, whilst the section on emergence sees this applied to describe the expansion of the host population and environmental niche. The ongoing outbreak in the Pacific northwest of North America is discussed, including some of its drivers. The section on adaptation highlights some insights into how eukaryotes adapt both to their environment and within hosts, and the final section covers the scientific response to this threat in terms of treatment and prevention. The review highlights both clinical and laboratory features of particular interest and places them into the wider context of this still emerging threat.

Keywords Cryptococcus · Emergence · Characterization · Adaptation · Environmental niche · Research

Cryptococcus spp. are considered ‘model yeasts’ by many researchers—the reasons for that are the topic of this review. Several perspectives will be discussed, starting with their characterization and emergence as human pathogens before moving onto their adaptations and the scientific response. The section on characterization illustrates the progress of knowledge and technology over the 120 years since *Cryptococcus* was first identified, whilst the section on emergence sees this applied to describe the expansion of the host population and environmental niche. The ongoing outbreak in the Pacific northwest of North America is discussed, including some of its drivers. The section on adaptation highlights some insights into how eukaryotes adapt both to their environment and within hosts, and the final section covers the scientific response to this threat in terms of treatment and prevention. It is beyond the scope of the review to cover every aspect of cryptococcosis, and the intention is just to summarize current knowledge of key topics and provide a starting point for further reading about this fascinating, complicated and important human pathogen.

A Model for Characterization

There are 70 species within the genus *Cryptococcus*. Two of these, *Cryptococcus neoformans* and *Cryptococcus gattii*, account for almost all cases of human disease and are amongst the most significant pathogenic yeasts in man, responsible for a large burden of mortality and morbidity [1–3]. They are widely dispersed throughout the environment; *C. neoformans* is associated with avian guano [4], and *C. gattii*’s first identified environmental niche was the Australian Red River gum tree [5, 6]—although both species have subsequently been isolated from a wide variety of trees [7–9]. Their global distribution and ability to cause disease are driven by the species

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or varietal form; the vast majority of cases occur in immunocompromised hosts, although disease in the immunocompetent is increasingly recognized [4, 10–12]. However, in general *Cryptococcus* spp. are not considered to be primary human pathogens; rather, the ability to cause disease is an accidental result of some adaptation to their usual saprophytic niche.

Speciation

Cryptococcus neoformans was first identified in 1894 following isolation from peach juice [13, 14] and almost contemporaneously identified as a pathogen in a patient with osteomyelitis [15]. Unlike other *Saccharomyces* spp., the organisms identified by Sanfelice and Busse did not ferment carbon sources nor form ascospores—and for this reason in 1901, they were transferred to the genus *Cryptococcus* [10]. The *Cryptococcus* genus consists of basidiomycetous saprophytes, remarkable for their polysaccharide capsule, which varies in size depending upon the environment in which the yeast finds itself. The capsule is a key determinant of virulence, and it is postulated that it enables survival of the yeast within free-living amoebae or other simple organisms. The capsule is easily identified on India Ink staining of clinical specimens; characteristic budding is often seen.

Pathogenic *Cryptococci* can be distinguished serologically. In 1935, Benham described serotypes A, B and C, using sera raised from inoculated rabbits [16]. The resolution of this method was improved by Evans in the 1950s [17, 18] enabling the identification of serotype D in the late 1960s and a hybrid form (AD) in the 1980s [19, 20]. *Cryptococcus neoformans* var. *grubii* generally types as A, *Cryptococcus neoformans* var. *neoformans* generally as D and *Cryptococcus gattii* types as B or C—but there are exceptions which make serological typing alone inadequate for accurate speciation [21].

Genotyping

Molecular typing techniques, notably restriction fragment length polymorphism analysis [22], amplified fragment length polymorphism analysis [23] and multi-locus sequence typing [24] have generally supported and clarified the pre-existing classification systems. However, they have also been instrumental in identifying new species, removing incorrect interspecies distinctions and informing the decision to raise *Cryptococcus neoformans* var. *gattii* from a varietal form to a species in its own right [10, 25, 26•]. Earlier observations that isolates were antigenically dissimilar, had different ecological niches and caused a different spectrum of disease (see below) have been validated by sequence-based technologies demonstrating that the two forms are evolutionarily distinct [27, 28], having diverged 40–80 million years ago [29, 30]. This progress demonstrates how advances in the technologies used for

classification have deepened the understanding of the genus and how this genus can be used as a model for progressive, systematic classification.

The polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis of the *URA5* gene has been particularly useful [22]. This simple tool divides *C. neoformans* into five genotypes (VNI-VNIV and VNB) and *C. gattii* into four (VGI-VGIV). The genotypes provide a basis from which to understand phenotypes, correlating with features such as outbreak epidemiology [10], antifungal susceptibility and virulence [31, 32]. However, recently, it has been noted that these genotypes have variable degrees of genetic heterogeneity, and some may benefit from subdivision (e.g. VGII being divided into a, b and c in the Vancouver Island outbreak—see below). Some genotypes may be sufficiently distinct to be classified as varieties, or even ‘cryptic species’—VG genotypes demonstrate distinctiveness at least equivalent to that seen between *C. neoformans* vars. *grubii* and *neoformans* [26•, 33, 34•]. Taxonomical debates continue as classification systems keep pace with developing technology [25, 35].

Metabolic Phenotype

Biochemical profiling can identify and distinguish *Cryptococcus* species, with the most obvious example being the use of Canavanine-Glycine-Bromothymol Blue (CGB) Agar to distinguish *C. neoformans* from *C. gattii*—because *C. gattii* is resistant to canavanine and can utilize glycine, its successful growth effects a change in pH which turns the medium blue, a pattern not seen with *C. neoformans* [36]. Other simple tests used for clinical identification include production of urease and melanin [4]. Commercial identification systems, such as API 20C AUX™ (bioMérieux SA, Marcy-l’Etoile, France) and Vitek™ (bioMérieux Inc., Hazelwood, USA) systems take advantage of species-specific carbohydrate utilization allowing cheap and rapid identification based on biochemical profiling [37].

Proteome analysis promises to offer increased insights into understanding the pathogenicity of complex organisms such as *Cryptococcus* sp., where post-translational modification may account for differences between strains [10, 38, 39]. Matrix-assisted laser desorption/ionization time of flight technology (MALDI-TOF) has made accurate quantification of protein components readily available to research and clinical laboratories. MALDI-TOF is cheap to perform, rapid and capable of high throughput [40]. Beyond use as a diagnostic tool, such analyses can demonstrate how virulence factor expression is affected by exposure to stressors such as temperature, radiation or chemicals [38, 41–43, 44•]. An example is how nitric oxide exposure results in upregulation of the antioxidant agents thioredoxin and glutathione and oxidoreductases involved in the yeast stress response and also results in

post-translational protein modification [42]. Mass spectral technologies have also been applied to biofilms, which play a role in cryptococcal virulence and have demonstrated that biofilm organisms upregulate proteins associated with defence (e.g. antioxidant, proteolytic or stress response proteins) whilst downregulating proteins associated with development (e.g. those responsible for translation, transcription and microtubule formation) [44•]. Key outputs hoped for from these new approaches are explaining mechanisms of drug resistance, identifying markers of resistance and identifying new drug targets [39, 45].

A Model for Emergence

Since its discovery, *Cryptococcus* has transformed from a rare pathogen to one estimated to infect one million and kill over 600,000 individuals each year [1]. There are no accurate estimates for the global incidence of non-HIV-associated cryptococcosis: sporadic cases in apparently immune-competent hosts persist [2, 46–50], but in addition, large outbreaks in immune-competent populations have recently been described and are discussed below. The common driving factor behind these areas of growth is exploitation of new niches, and the most dramatic example of this is the HIV pandemic, which continues to be the commonest risk factor globally. However, in rich countries, ongoing emergence is increasingly driven by non-communicable causes of immune deficiency [2, 46, 47, 50–52] including iatrogenic immunosuppression due to solid-organ transplants or biological immunomodulatory therapies [52–54].

Emergence of *Cryptococcus neoformans* as an Endemic Pathogen

Recent developments in the understanding of the origin of the AD hybrid serotype have led to the postulation that the evolutionary source of *C. neoformans* is Africa. *Cryptococcus sp.* has two mating types, α and a, and can exist in haploid or diploid forms. In its haploid form, it demonstrates a single mating-type allele (' α ' (MAT α) or 'a' (MATa) with the diploid forms expressing one or both alleles (aa, $\alpha\alpha$, a α). Only haploid cells mate, typically attracting their opposite mating type with the secretion of pheromones, developing projections and fusing to produce diploid progeny, which then quickly divide by meiosis into four new haploid organisms, with varying degrees of recombination [4]. For convenience, these forms are annotated as follows: for haploids, serotype X, mating-type y (Xy); for diploids yXXy. So, serotype A with the MAT α mating-type allele (the commonest combination globally [4]) would be A α ; serotype AD, with A carrying the MATa mating-type allele and D the MAT α , would be aAD α .

Interest in the origins of the AD hybrid resulted from the anomalous fact that almost all global isolates of serotype A demonstrate the MAT α (A α) mating-type allele [4], yet serotype AD, which is also globally distributed, frequently demonstrates the serotype A MATa mating-type allele (aAD α) [55]. Various explanations were proffered, including the idea that Aa became extinct overtime and that the aAD α hybrid predated this extinction. Another suggestion was that Aa had an unidentified environmental niche or lacked virulence and so was seldom isolated due to sampling bias.

However, the discovery of a genetically distinctive serotype A (VNB genotype) subpopulation in Botswana raised new and intriguing possibilities [24, 55]. The proportion of *C. neoformans* var. *grubii* with the MATa allele in Botswana is around 25 %, compared with a global proportion of less than 0.1 %, and the population structure shows a high degree of heterogeneity not seen in other geographic regions [24]. In 2007, Litvintseva and colleagues used a combination of AFLP- and MLST-based phylogenetic analyses to show that the Botswanan Aa was very closely related to the Aa genes in global aAD α hybrids from China, Europe and the USA [55]. They then considered the impact of hybridization on fitness, using UV irradiation as a stressful event and comparing the response of Botswanan Aa and A α isolates, with reference A α isolates, lab-generated aAD α hybrids, wild-type aAD α hybrids and reference serotype D isolates. Botswanan Aa isolates were more sensitive to UV radiation than any other isolate, and hybridization with D conferred significant UV resistance [55]. These two pieces of information form the basis of the hypothesis that a large proportion of the global AD population resulted from a hybridization event in Botswana, producing a fitter organism, which was able to go on and colonize the world.

Colonization may have been facilitated by a combination of the great human migrations, expansion of domesticated pigeon populations and the huge expansion of international trade in Africa driven by European imperialism [55–60]. This theory has been further supported by research in Thailand, where a genetic bottle neck, analogous to a founder effect, was identified in *C. neoformans* var. *grubii* isolates [57]. Strong signatures of clonality were detected, with the null hypothesis of random recombinations being rejected, in stark contrast to the genetic heterogeneity seen in African isolates [57]. The mean time to the most recent common ancestor was estimated to be approximately 7000 years ago, well after the currently estimated dates for the human out-of-Africa migration [58, 61, 62], suggesting *Cryptococcus* left Africa with man, pigeons and perhaps other vectors to ultimately expand clonally in new geographic locations. This ex-African clonality has been described in multiple other geographic locations, and is believed to demonstrate an epidemic population structure for the organism [12, 24, 63, 64].

Emergence of *Cryptococcus gattii* as an Agent of Outbreaks

Cryptococcus gattii is estimated to have diverged from *C. neoformans* approximately 40 to 80 million years ago [29, 65]. It was first described in 1970 in a Congolese patient with cryptococcosis; the infecting isolate was found to have a distinctively different morphology. Instead of the usual uniform round or oval forms seen with *C. neoformans*, this new isolate produced frequent elongated or bacilliform morphotypes [66, 67]. Subsequently, a number of human cases were described in Australia, and pioneering work was undertaken to identify its environmental niche [6, 68]. Following extensive sampling of plants, their debris, soil and air, the major niche was identified as the Red River Gum, *Eucalyptus camaldulensis*. Since then, numerous other tree species have been identified as habitats; the particular niche appears to be rotting bark and wood, as well as soil beneath the canopy [8, 9, 34, 69–73]. Historically, the incidence rates of *C. gattii* cryptococcosis have been highest in Papua New Guinea (42.8/million/year) and Australia's Northern Territory (8.5/million population/year) [74]. Unlike disease due to *C. neoformans*, patients diagnosed with *C. gattii* usually have no identified immune deficiency, and pulmonary involvement is more common [75, 76]. Host factors may have an impact on the risk of disease—in Australia, the incidence rate in the aboriginal population is 10.4/million/year compared to the non-indigenous population rate of 0.7/million/year, and the difference is not thought to be wholly explained by differences in geography [74].

It was previously believed that *C. gattii* was limited to the tropics and subtropics, but it is increasingly being recognized in temperate regions. The ongoing outbreak on Canada's Vancouver Island and in the Pacific northwest of the USA provides an excellent illustration of a pathogen exploiting a new environmental niche; incidence there is now 25.1/million/year [77].

The various *C. gattii* genotypes differ in their global distribution, reproductive behaviour and pathogenicity. The VGII genotype appears to be the oldest, estimated to have diverged from a common ancestor 12.5 million years ago; VGIV diverged 11.7 million years ago; and VGI and VGIII diverged from each other 8.5 million years ago [29]. VGI is the genotype most prevalent in Australasia and Europe, VGII is most prevalent in South and North America (including the current Pacific North West outbreak), VGIII is more common in North and South America than other regions (but not predominant), and VGIV is the most frequently described in Africa [26, 78]. The genotypic spread of *C. gattii* and *C. neoformans* is depicted in Fig. 1. The data (review by Cogliati, 2013) contains a combination of human, veterinary and environmental samples from 2012 and earlier which were not necessarily collected under formal surveillance nor randomized sampling

programmes. Despite these limitations, it is interesting to note the regional variations, especially in *C. gattii* [79].

Vancouver Island Outbreak

Since 1999, there has been an outbreak of *C. gattii* disease centred around Vancouver Island, Canada, and by 2009, it had spread to northwestern USA [80]. This outbreak has largely resulted from clonal expansions of three subtypes of VGII (VGIIa, VGIIb and VGIIc). VGIIa dominates, and is termed the 'major' strain. Laboratory models suggest it has enhanced virulence—it replicates rapidly within macrophages, with a high intracellular proliferation rate (IPR), and leads to shorter survival times in the mouse infection model. Interestingly, this increased virulence may in part be explained through mutations in its mitochondrial genome [32, 81]. Voelz et al. recently clarified the previously described link between VGIIa's increased IPR and its capacity to transform its mitochondrial morphotype to tubular (from globular) [81]. It had already been noted that a tubular mitochondrial morphotype was commoner in the pathogenic outbreak strain [32]. Using time-lapse images, it was noted that VGIIa cells can rapidly tubularize their mitochondria in response to oxidative stress, becoming significantly less likely to be killed by the macrophage and yet slower to replicate than those with globular mitochondria. The finding of reduced fecundity, in a strain with a higher IPR, was explained by the observation that the remaining yeast cells (with globular mitochondria) replicated very rapidly. In the presence of the resistant but non-replicative VGIIa tubular mitochondrial morphotype, even *non-outbreak* strains are stimulated to increased IPRs, suggesting a signalling pathway whereby yeast cells establish a 'division of labour' [81]. This may have implications for other infections and especially co-infections occurring in the presence of *C. gattii* [81].

VGIIb, which is termed the 'minor' strain, demonstrates less virulence than VGIIa in both in vitro and in vivo models [32, 82], although a difference in human outcomes has been harder to demonstrate [77]. VGIIb is responsible for less than 10 % of cases in this outbreak, and because many of those affected are in older age groups, it is difficult to compare clinical outcomes [77]. VGIIc is similar to VGIIa genotypically and phenotypically but is unique to the USA. It was first isolated in Oregon and appears to be the result of a recombination event, either locally or prior to import [32]. Currently in Oregon, VGIIc causes 27 % of infections and VGIIa approximately 63 % [83], compared to 0 and 86.3 %, respectively, in British Columbia [77].

The origin of the outbreak strains has been the source of debate. Until recently, evidence was balanced between the likeliest candidates: South America, Africa and Australia (which all had evidence of recombination and genetic diversity). However, recently, the case for South America has

Fig. 1 Paired pie charts representing the genotypic distribution of *C. neoformans* (left) and *C. gattii* (right) in North America, South America, Europe, Africa, Asia and Oceania. The size of each chart represents the number of genotyped isolates in the analysis for that region, out of a global total of 8077. Data from Cogliati 2013 [79]

strengthened [29], due to the discovery of a strain displaying a basal genetic lineage in virgin Amazonian rainforest, where contamination from imported woods is thought extremely unlikely [84].

Ultimately, it is hoped that better understanding the relationships between subtypes and the environmental niche will enable the public health community to predict the likely range of current outbreaks and regions at risk of outbreaks in the future [85].

A Model for Adaptability

Virulence in Animal Hosts Results from Adaptation for Survival in the Environment

Cryptococcus spp. are primarily environmental saprophytes. Human-to-human transmission does not occur, and therefore adaptive pressures that confer pathogenicity must come from the non-human environment. It is widely held that the ability to cause disease is due to the coincident pathogenic potential of adaptations to its local environment: so-called ‘bystander pathogenicity’ [86, 87]. *C. neoformans* possesses a variety of specialized virulence factors, including an external polysaccharide capsule, the ability to grow at mammalian body temperature, production of laccase and melanin, and a number of secreted factors (including phospholipase B and urease [88]) important for surviving the host immune response [10]. Although *Cryptococcus* spp. can infect hosts ranging from amoebae to mammals, there is no evidence that a susceptible host is required at any stage of the life cycle. Even pigeons, considered to have an important role in the global dispersal of *C. neoformans*, are not susceptible to cryptococcosis so are unlikely to exert evolutionary pressure [4, 89]. So, given the myriad interactions between *C. neoformans* and other environmental organisms, current data suggest that selection pressures from environmental stress and predation have led to the

acquisition of a unique set of cryptococcal attributes, which just happen to be useful in mammalian infection, rather than there being some obligatory stage within a (as yet unidentified) mammalian host. Some of these attributes are described below.

Uptake and Subsequent Intracellular Proliferation

Ruiz et al. (1982) observed that 99.9 % of *C. neoformans* could be ingested and killed by *Acanthamoeba palestinensis* trophozoites after 7 days and that it could also be ingested by mites and sowbugs—all found in pigeon guano [90]. Ingestion of *C. neoformans* by *A. castellani* is followed by intracellular replication and the accumulation of cytosolic vesicles containing shed capsular polysaccharide [91]. Replication is impaired in mutants with deficient capsules or phospholipase, and acapsular mutants are more readily killed after ingestion [92, 93]. Melanin production may also protect against amoeba predation since, even in acapsular strains, melanized individuals are more likely to survive ingestion [92, 93]. Thus, factors associated with virulence in mammals may have evolved to fulfil a role in cryptococcal interactions with amoeba.

The interactions of *C. neoformans* with amoeba and host macrophages are strikingly similar. In mammals, *C. neoformans* is a facultative intracellular pathogen which can survive phagocytosis by innate immune cells and proliferate intracellularly [10]. The development of such transferable intracellular survival skills may help to explain the broad host range of *C. neoformans*: all major mammalian immune responses rely on phagocytic effector cells. Following invasion of the phagocyte by *Cryptococcus*, there may be dissemination by non-lytic extrusion of yeast cells to both the extracellular space and adjacent host cells (‘vomocytosis’) [92, 94–99]. This potential ‘Trojan horse’ effect may be the key in establishing central nervous system infection [100–102]. *Cryptococcus*’ complex pattern of interaction with host innate

immune cells is a key determinant of virulence—both intracellular proliferation rate (IPR) and phagocytosis proclivity have been correlated with mortality for *C. gattii*, and an enhanced ability to survive and replicate inside macrophages has been shown to be associated with its spread in northwestern North America and may even have triggered the outbreak [98, 101, 103–105].

For *C. neoformans*, in contrast to *C. gattii*, it seems initial yeast uptake by macrophages, rather than IPR, correlates with cerebrospinal fluid (CSF) fungal burden [103]. Infection with high-laccase activity, high-uptake *C. neoformans* correlates with higher pre-treatment fungal burden in patients' CSF and slower rates of fungal clearance with treatment [103]. In this case, strains with smaller capsules are more readily engulfed by macrophages and have enhanced intracellular survival.

Capsule

C. neoformans' capsule is unique amongst human pathogenic yeasts and is comprised of polysaccharides (glucuronoxylomannan (GXM) and galactoxylomannan (GalXM)) and a lesser proportion of mannoproteins (MP)) [106]. In addition to the effects on phagocytosis by amoebae described above, the capsule may also protect against dehydration and desiccation in low humidity conditions [107].

The role of the capsule in pathogenesis and defence against the mammalian immune response is well established [106, 108, 109]. Acapsular *C. neoformans* strains have reduced pathogenicity in mice; the capsule promotes survival in their lungs, dissemination to the brain and induces a more subdued cellular response [110, 111]. The capsule also protects unopsonized yeasts from ingestion by phagocytes in vitro, depletes complement components and impedes immune response [112]. Capsular polysaccharide materials induce proliferation and differentiation of normal CD4⁺ T cells into Th2 phenotype, favouring intracellular parasitism and dissemination [113]. The fact that acapsular *C. neoformans* strains are only capable of causing prolonged brain infections in nude mice (absent or deficient thymus) demonstrates the key role of capsule in virulence [4]. Despite this, links between capsule size and virulence were not demonstrated until recently when a paper by Robertson et al. showed a larger ex vivo capsule size was associated with higher opening CSF pressure and a paucity of CSF inflammation [109]. They also showed that the larger capsule size was associated with more shedding of capsular antigens and viscosity of the CSF, which may be a mechanism for the elevated CSF pressure [109].

Growth at 37 °C

The ability to grow at mammalian body temperatures (37 °C or higher) is an obvious yet essential attribute for any would-be invasive fungal human pathogen [114–117]. There are

several explanations for the origin and evolution of thermotolerance in *C. neoformans*. It has been hypothesized that a bolide collision 65 million years ago eliminated a vast proportion of the Earth's fauna and triggered a global chain reaction of volcanic and seismic activities, giving rise to a massive fungal bloom that fed on decaying vegetation [114]. Under these conditions, endothermy was a costly but effective adaptation for surviving mammals and birds species to offer protection against such a large fungal inoculum. Fungi would have to adapt too to successfully infect, colonize and cause disease in these new endothermic hosts [114]—however, this adaptation would not be critical for an organism that does not require mammalian hosts, so adaptations to warmer environments may offer a likelier explanation.

Strains of *C. neoformans* have been shown to be able to grow in a temperature range of 30 to 40 °C [117], but there is a considerable variation in thermotolerance between different *C. neoformans* strains, and some evidence that this is determined by geographic origin. For instance, *C. neoformans* var. *neoformans* strains, which in general are more susceptible to high temperature than their globally distributed *C. neoformans* var. *grubii* siblings, are more prevalent in temperate regions of Europe [118]. Evidence that *Cryptococcus* spp. originated in sub-Saharan Africa further supports the speculation that growth at higher temperatures, essential for pathogenicity, is a survival advantage that resulted from environmental selection pressures in regions with higher ambient temperatures [7, 119].

Laccase and Melanin Production

Melanin is a brownish black pigment produced through oxidative polymerization of phenolic compounds. *C. neoformans* produces melanin with an oxidative enzyme called laccase which synthesizes melanin from L-DOPA, dopamine, norepinephrine and epinephrine [120–122]. Environmental isolates of *C. neoformans* are often melanized; melanization may have evolved as a survival strategy to protect cells from UV radiation and facilitate growth at extreme temperatures [123–125]. Defects in melanin production result in improved mouse survival in infection models [122] and have been shown to protect against enzymatic degradation, antimicrobial peptides, oxidative stress and heavy metal toxicity [126, 27–129]. Of note, it also decreases the efficacy of amphotericin B in vitro [128]. Moreover, clinical isolates with higher laccase activity have higher ex vivo CSF survival and are more resistant to antifungal treatment [103].

Cryptococcus also Displays In-Host Adaptations

Morphology Switching

Cryptococcus spp. are capable of morphological transformations, and these can occur during infection. Such

transformations include filament formation during mating, and upregulation of capsule synthesis to produce giant cells averaging 40–50 μm in diameter [93, 106, 130, 131] (see Fig. 2), but up to 100 μm [132]. In mouse models, the giant cell subpopulation can vary between 10 and 80 % of the total pulmonary fungal population, depending on the duration of infection, degree of inflammation and total fungal burden [93]. Mice infected with a lower dose of yeasts, with less inflammation, produce a higher proportion of giant cells [93]. These giant cells are often polyploid but uni-nucleated, suggesting DNA replication without subsequent completion of mitosis and/or cytokinesis. This variable chromosomal ploidy in the giant cell population indicates both dynamic flexibility and stability of the cryptococcal genome [88, 93, 132].

Pheromone and cyclic AMP-dependent signalling pathways have been shown to be major regulators of cryptococcal cell gigantism [88, 93, 133–135]. Temperature has been identified as a stimulus for this change in some strains [136]. The formation of giant cells may be a survival strategy facilitating evasion of host immune defences: giant cells are frequently found in extracellular spaces and are more resistant to phagocytosis than regular yeast cells, perhaps representing an extracellular subpopulation, co-colonizing with an intracellular population of smaller cells which have utilized phagocytosis to facilitate dissemination. Lower CSF fungal burdens in patients with chronic or latent (extra-CNS) infections are in keeping with the observation that lower inocula seem to induce more gigantism [88], leaving fewer regular cells to hijack macrophages to gain access to the CSF. Morphological heterogeneity presents an obstacle for the immune response, which may explain some of the observed difficulties in cryptococcal disease therapy [129].

Fig. 2 India ink staining of a Vietnamese clinical isolates of *Cryptococcus neoformans* var. *grubii* showing giant cells alongside standard-sized counterparts. Image courtesy of Lam Tuan Thanh, OUCRU, Vietnam

Genome ‘Flexible Stability’ and Survival-Driven Adaptability

Early characterization of cryptococcal genomes came from electrophoretic karyotyping [137]. Both the size and number of chromosomes were highly variable in clinical and environmental isolates, suggesting extensive chromosomal rearrangement and genomic flexibility [138]. Further investigation revealed the extent of variation within the population and suggested meiotic-driven karyotypic variation [139, 140]. Karyotype changes also occur within a single infection; this was observed in a series of human cases and confirmed in the mouse model [141]. Gross chromosomal rearrangement has been observed in closely related strains isolated from the same patient 77 days apart, further supporting host-selective pressures as a driving force behind this genome flexibility [142–144].

Full genome sequences became available for *C. neoformans* var. *grubii* in 2001 and *C. neoformans* var. *neoformans* in 2005. Despite karyotypic variability, these closely related varieties share over 80 % sequence homology and are largely collinear [145] though not at the subtelomeric regions, centromere or MAT loci [146]. The retention of such similarity is remarkable given their separation over millions of years, and the combination of genomic stability with the capacity to undergo extensive chromosomal rearrangement upon selective pressure is something of a paradox [142].

This genomic flexibility is likely a response to within-host pressures and could be considered a virulence-associated phenotype with possible consequences for treatment. For example, the capacity for aneuploidy may be a key factor in conferring resistance to fluconazole [147–149]. This phenomenon is seen in *Candida albicans*, where chromosomal rearrangement and duplication is proposed as a mechanism allowing populations to respond to selective pressures [150]. This has been termed heteroresistance, whereby a subpopulation of resistant organisms exists amongst a larger population of susceptible siblings, thanks to some degree of genomic plasticity. Various studies on azole heteroresistance in *C. neoformans* have demonstrated disomy in chromosomes 1 and 4 occurring in resistant subpopulations selected from growth conditions containing high antifungal drug levels [147, 151]. Resistant clones emerging in media with the highest drug levels were found to have disomies in four chromosomes: Chr1, 4, 10 and 14 [147, 151]. Amplification of ATP transporter efflux pump genes like *Erg11* and *Afr1* on Chr1 was shown to correlate with azole resistance [147], and disomy on Chr4 has been associated with genes required for endoplasmic reticulum integrity under azole stress [151]—there may be other mechanisms to be discovered.

A Model for Scientific Response

Medical research into the appropriate management of cryptococcal disease has increased rapidly since the advent of the HIV epidemic [1]. Unlike many CNS infections, cryptococcal meningitis is relatively easily diagnosed, with high fungal burdens and a distinctive pathogen that can be seen with a number of non-specific staining and culture methods or identified with highly sensitive antigen detection kits [4]. However, outcomes remain unacceptably poor—with a 90-day case fatality rate of up to 70 % [1, 3, 77, 152•]. Even when best available treatments are provided in well-resourced settings, mortality is upwards of 10–15 % [3, 77].

Antifungal Therapy—a Model for Clinical Research

Amphotericin B was the first treatment available for cryptococcosis and became available in the late 1950s [4]. The arrival of fluconazole in the 1980s, with its excellent oral bio-availability and tolerability, revolutionized treatment enabling the shortening of duration of amphotericin treatment and the long-term suppressive therapy required by immune suppressed patients. There have been almost 40 randomized controlled trials of antifungal therapy in cryptococcal disease, although in general, these have been small (the median number of patients randomized is 58), and have not been powered to survival (see Table 1). Rather, trials tended to use composite endpoints consisting of survival, clinical improvement and sterilization of cerebrospinal fluid. A major focus for the first trials was testing increased doses of amphotericin, shortening treatment regimens and combination treatment with flucytosine [153–155].

A landmark paper was the 1997 AIDS Clinical Trials Group and Mycoses Study Group study of 379 participants, comparing treatment with higher dose amphotericin monotherapy to flucytosine combination therapy. This established

the amphotericin–flucytosine combination as the gold standard for HIV-infected patients with improved clinical and mycological outcomes; however, the trial lacked power to demonstrate a survival effect [155]. Combination therapy has formed the backbone of treatment recommendations since then [156]. In 2013, the *survival* benefit was demonstrated in a large trial from a single centre in Vietnam [152•]. Flucytosine also leads to improved outcomes and more rapid fungal clearance when combined with fluconazole (compared to fluconazole monotherapy), although studies have been small [157–159]. Unfortunately, access to both amphotericin, and in particular flucytosine, is extremely poor in the locations where most disease occurs [160]. Direct comparisons of non-amphotericin containing combination treatments with amphotericin-based combination treatments are underway [161].

In settings where flucytosine is not available, the combination of amphotericin with fluconazole is recommended [156], although there is no clear evidence that this improves mortality. Patients receiving 2 weeks of induction treatment with amphotericin combined with fluconazole had similar outcomes at 10 weeks and 6 months to patients receiving 4 weeks of amphotericin monotherapy [152•].

Trials in cryptococcal disease are challenging. HIV-infected patients are complex with multiple health needs and require prolonged follow-up; this in part explains the relative paucity of data to inform treatment decisions. The development of early fungicidal activity (EFA) as a measure of treatment effect offers the chance to speed treatment development. Following its conception in Thailand and its subsequent use in numerous intervention studies, there is hope that it will eventually serve as a robust surrogate marker of the efficacy of antifungal treatments [162–167]. Differences in EFA provide biological plausibility for the differences in survival seen with different treatments in the Vietnamese study [152•].

Table 1 Cryptococcal trial summary statistics as of 1979–2014

Factor	Value
Estimated number of cases of CM per year in HIV patients ^a	957,900
Estimated number of CM deaths per year ^a	624,700
Number of patients with CM randomized in treatment trials	3182
Studies powered to mortality	1
Studies that have shown a mortality difference	3
Number of randomized controlled trials (RCTs) in cryptococcal meningitis	35
Median number of patients per RCT	58
Percentage of trials with less than 100 patients	75
Percentage of trials in HIV patients	92
Median days from receipt of funding to randomizing first patient in a trial ^b	611

^a Estimates from Park et al, AIDS 2009

^b Refers to any clinical intervention trial in any disease

Adjuvant Therapies

Because of a lack of new antifungal agents, several groups have undertaken research seeking options for adjuvant therapies including corticosteroids [168], IFN- γ [169, 170•] and acetazolamide [171]. Results from the corticosteroid trial are yet to be published, IFN- γ has been shown to have a statistically significant impact on EFA (but not mortality) and the acetazolamide trial was discontinued early due to an excess of adverse events, likely due to additive toxicity with amphotericin. Currently, none of these adjuvants can be recommended in routine clinical care.

The timing of initiation of antiretrovirals (ARVs) in HIV co-infection has been a key area of interest over recent years, with a need to balance the benefits of ARVs (especially with regards to opportunistic infections) with the possible harmful consequences of immune reconstitution syndromes. In pulmonary tuberculosis, early initiation appears to be beneficial, although this effect was not seen in a randomized trial in TB meningitis [172–175]. In cryptococcal meningitis, the COAT trial (2014) found that early initiation of ARVs was associated with an increased risk of death and appeared to carry no benefit in terms of reduced incidence of opportunistic infections [176•]. Previous smaller studies also suggested probable increased risks of harm with early initiation of ARVs [177, 178]. It is hypothesized that the increase in mortality is related to immune reconstitution inflammatory syndrome (IRIS) and that the impact is greater earlier in the course of treatment, when the patient has not yet begun to recover from the original intracerebral insult and has limited reserves. In keeping with this theory, Boulware et al. noted that the increased mortality was especially pronounced in patients with a baseline CSF white cell count <5 cells/mm³ [176•], an established risk factor for IRIS [179, 180].

Perhaps the most valuable adjuvant in the treatment of cryptococcal meningitis is the management of raised intracranial pressure. International guidelines recommend that pressure should be maintained below 25 cm of CSF, with daily lumbar punctures if necessary [181]. Pressure is elevated at presentation in over half of patients [152•, 176•, 182], and failure to comply with management guidelines has been associated with adverse outcomes [183]—fears about frequent lumbar punctures leading to harm appear to be unfounded [184, 185]. However, there have not been any prospective trials comparing different pressure management methods.

A Model of Early Detection and Treatment

Current antifungal and adjuvant options have yet to achieve the large reductions in mortality required in cryptococcal

meningitis but perhaps an upstream approach could. Early diagnosis is invariably beneficial in the management of infectious diseases, and there is much interest in the use of lateral flow antigen (LFA) detection test for cryptococcal antigen (CrAg) to diagnose a pre-symptomatic stage of cryptococcal meningitis where treatment efficacy may be improved. Undoubtedly, the LFA test brings opportunities to improve standard diagnostics in resource-limited settings [186], as it requires little equipment, no electrical power and minimal training. CrAg positivity has been shown to predate symptoms of cryptococcal meningitis by several weeks [187] and is associated with increased mortality [188, 189]. Modelling suggests that systematic screening and treatment could be a cost-effective intervention in selected patient populations [190–192], and screening of asymptomatic patients is being introduced in South Africa on a country-wide scale. However, the best treatment for asymptomatic antigenaemia is unknown. A prospective observational cohort suggested a benefit of pre-emptive administration of fluconazole in such patients, but dosing schedules were uncontrolled and varied considerably, preventing the formulation of a treatment recommendation [190]. Further work in this area is urgently needed. Another area of concern is illustrated in a study from Cambodia which showed that many outpatients with antigenaemia, without meningism, had evidence of disease on CSF examination [193]. An acceptable CrAg screening strategy would need to ensure patients with cryptococcal meningitis (CM) continued to receive evidence-based therapy.

A simplified but common format for a screen and treat approach is depicted in Fig. 3 [194•]. Importantly, it acknowledges that optimal treatment for asymptomatic antigenaemia has not been established and that lumbar puncture should be performed whenever feasible to exclude CM [195]. Such a programme was predicted to be cost-effective in a Ugandan study [190], and two studies in South East Asia suggested implementation would cost less than 300 USD per life year gained (depending on actual prevalence of CrAg positivity, and fluconazole drug costs)—even at the top end of this range, the intervention would be classified as ‘very cost-effective’ under WHO guidelines [191, 192].

However, when implemented in an ambulatory care setting in Kenya, 782 patients were enrolled into the screen and treat intervention and offered high-dose fluconazole followed by a maintenance dose if they tested positive. Unexpectedly, the study failed to demonstrate any overall mortality benefit to the intervention. The study was compromised by poor uptake of the intervention and the fact that historical controls were used, but in this ‘real world’ setting, there was little to suggest any benefit [196•]. A different approach in Tanzania, where a universal screening of hospitalized HIV patients was undertaken, failed to improve diagnostic accuracy beyond standard of care and did not demonstrate a mortality benefit [197]. In both studies, due to local resource issues, all patients received

Fig. 3 Proposed screen-and-treat algorithm for cryptococcal antigenaemia [194•]

fluconazole monotherapy even if meningitis was diagnosed—mortality was high in CM patients, and inadequate drug therapy may have masked any mortality gains from early diagnosis, even though the proposed benefit from preventing cases of CM should have been realized.

Cryptococcal infections are challenging to manage, and there is a value in considering the story of the scientific response when considering how to respond to future emerging infections. Firstly, the diagnostic tests and treatments must be available where the burden of disease is highest, and ideally, research should be undertaken in these settings [198]. Secondly, trials should be powered for relevant clinical endpoints until surrogate endpoints have been conclusively validated. Finally, it shows the importance of having proposed preventative medicine interventions such as early initiation of ARVs and pre-symptomatic screening trialed in a robust, real-world setting before they are rolled out, as results can be unexpected.

Conclusion

Cryptococcus spp. are considered model yeasts for a variety of reasons: disease is relatively easy to diagnose; a wide-variety of laboratory models of infection have been developed; organisms are convenient to keep and grow in laboratory environments; and they are well characterized as a result of their major public health impact and disease is severe, so the impact of an intervention is readily measured.

Cryptococcosis remains a challenging condition which is unlikely to go away whilst populations of iatrogenically immunosuppressed hosts increase and the pathogen itself continues to adapt to new environments and hosts. The high mortality associated with best available treatment demands that further therapeutic trials are undertaken with new antifungals or adjuvants. Adaptive trial designs and international

collaborations should allow these to be powered to clinically relevant endpoints until such a time as surrogate endpoints have been validated.

A further ongoing focus should be systems-based approaches to earlier diagnosis, such as LFA screening—but these must be clearly evaluated for benefits in real-world settings. As cryptococcal disease continues to emerge; ongoing research should see the development of models and approaches likely to be transferable to other pathogens in the future. Monitoring the trajectory of its adaptations to new environments and new hosts will allow health care planners to consider its likely future impact. Together, these measures will hopefully address the unacceptably high global mortality and morbidity associated with this model CNS pathogen.

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