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Cryptococcus gattii: an emerging fungal pathogen infecting humans and animals

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Abstract

Infectious fungi are among a broad group of microbial pathogens that has and continues to emerge concomitantly due to the global AIDS pandemic as well as an overall increase of patients with compromised immune systems. In addition, many pathogens have been emerging and reemerging, causing disease in both individuals who have an identifiable immune defect and those who do not. The fungal pathogen *Cryptococcus gattii* can infect individuals with and without an identifiable immune defect, with a broad geographic range including both endemic areas and emerging outbreak regions. Infections in patients and animals can be severe and often fatal if untreated. We review the molecular epidemiology, population structure, clinical manifestations, and ecological niche of this emerging pathogen.

Keywords

Fungal infections; Cryptococcosis; Emerging outbreak; Pulmonary disease; Meningitis

1. Introduction

Infectious diseases cause a significant burden on human and animal health, plants and agriculture, and the global economy [1–3]. Among the microbial pathogens causing infectious diseases worldwide, fungi represent one major class. Advances in the healthcare of patients with noninfectious diseases coupled with the rise of HIV/AIDS globally, and continued outbreaks of fungal pathogens in otherwise healthy hosts, have led to an overall increase of fungi as etiologic agents producing human disease. Each of the four major fungal phyla has representatives that cause serious disease in both humans and a vast range of other animals. Although less prevalent than plant fungal pathogens, the animal fungal pathogens pose serious threats to entire animal populations (e.g., *Batrachochytrium dendrobatidis* in amphibians [4], *Geomyces destructans* in bats [5], and *Nosema ceranae* and/or *Nosema*-iridovirus co-infections in honey bees [6–9]) and continue to cause serious morbidity and mortality among immunocompromised humans and even individuals with no apparent immune defect worldwide.

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In many cases, the incidence of fungal disease is increasing due to the rise in susceptible hosts. Furthermore, limited fungicidal treatment options exacerbate both morbidity and mortality. A major factor influencing the lack of effective and safe treatment is that, unlike bacteria and viruses, the fungi are eukaryotic siblings to the animals and share several conserved signaling cascades. These issues cause major obstacles in the search and development of new antimicrobials that target fungi without causing major toxic side effects in the host. Several fungal species frequently cause infections in immunocompromised human hosts (e.g., *Candida albicans*, *Aspergillus fumigatus*), while others are known to cause disease in otherwise healthy individuals (e.g., *Coccidioides immitis*, *Coccidioides posadasii*, *Histoplasma capsulatum*). The pathogenic *Cryptococcus* species complex, consisting of *C. neoformans* and *C. gattii*, causes disease in both of these populations [10,11]. This is particularly evident with *C. gattii*, which is endemic in many tropical and sub-tropical regions and has also been associated with outbreaks in humans and a wide range of mammals, making it of global public health interest [10–12].

C. gattii is a basidiomycetous yeast and is estimated to have diverged from *C. neoformans* ~37.5 million years ago [13]. In aggregate, pathogenic *Cryptococcus* species produce disease in almost one million individuals annually with over 620,000 attributable mortalities and account for ~1/3 of all HIV/AIDS associated deaths, surpassing tuberculosis mortality in Africa [14]. *C. gattii* has a distinct epidemiology from its sibling species *C. neoformans*, which more commonly causes disease in immunosuppressed hosts. Within the species, *C. gattii* can be further subdivided into two serotypes (B and C) [15], and four molecular types designated VGI, VGII, VGIII, and VGIV [16]. Based on phylogenetic analyses, each VG group appears to show no inter-molecular type nuclear genetic exchange, indicating that the four molecular types are cryptic species, which could be assigned their own species designations [17–23]. Molecular type discrimination is of principal importance clinically and epidemiologically, as types VGI and VGII have been associated with the majority of cases from otherwise healthy hosts with outbreaks in the North American Pacific Northwest, in the Northern Territory of Australia in Aboriginals, and in the central province of Papua New Guinea. On the other hand, molecular types VGIII and VGIV appear to more commonly produce disease in immunocompromised patients including those with HIV/AIDS with documented case series in Africa and the United States. In fact, molecular types VGIII and VGIV appear to be similar to the epidemiological profiles observed for the sibling species, *C. neoformans* (Table 1) [10,11,14,24–28].

Overall, recent epidemiological studies of *C. gattii* suggest that this species is an emerging fungal pathogen with an expanding geographic range and environmental niche. In nature, *C. gattii* reproduces asexually as a budding yeast (Fig. 1A) and sexually to produce infectious spores (Fig. 1B); it is likely that the inhalation of both desiccated yeast cells and spores cause infections. While infections remain relatively rare, the level of mortality, percentage of patients with prolonged hospital visits, lengthy treatment regimens, and long-term sequelae remain common among infected patients [11,29,30]. Furthermore, this fungal disease can occur in a range of domestic, agricultural, wild terrestrial, and marine mammals [20,23,31–40]. To better understand the dynamics of these infections from a global perspective, a multidisciplinary approach is necessary. For these reasons, aspects of molecular biology, ecology, molecular epidemiology, traditional epidemiology, clinical practices, veterinary treatments, and therapeutics must be bridged together with each respective specialty aware of the basic tenets and principles of the current research in each respective field. In this review, sections are divided into specific areas of focus, with the aim of broadly summarizing important findings and future directions for this emerging/re-emerging infectious disease. Additionally, one section exclusively covers key aspects of the North American Pacific Northwest outbreak, as it represents the largest primary outbreak of *C. gattii* reported, and has emerged in an environmental climate that had not been previously

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associated with this disease and supports new features to the epidemiology of this encapsulated yeast [17,19,20,23,24,29,32,41].

2. Molecular typing methods

As previously mentioned, *C. gattii* can be divided into four discrete lineages (i.e., molecular types) termed VGI–VGIV [17,18,42–44]. This is significant as each molecular type has distinct characteristics. Molecular types VGI and VGII are the two types most frequently associated with illness in otherwise healthy individuals [24]. Infections due to VGI have been reported to occur at high rates among certain populations in Australia such as Aboriginal groups but there have also been numerous cases elsewhere globally. In contrast, the numbers of VGII infection are high in the Pacific Northwest, in which over 95% of all cases are attributable to this molecular type [11,17,18,24,45]. In comparison, molecular types VGIII and VGIV have largely been associated with illness in HIV/AIDS patients, which is similar to the epidemiological profile observed for *C. neoformans* [10,11,14,24,25]. Molecular type VGIII has been isolated from a number of regions worldwide [17,18], and was found to be the predominant molecular type (>93%) in a cohort of HIV/AIDS patients infected with *C. gattii* from Southern California [46] (Byrnes et al., *PLoS Pathogens*, in revision). Molecular type VGIV appears to be rare in the global population but has been reported among HIV/AIDS patients in sub-Saharan Africa [25]. Overall, these distinctions are significant as there are clear associations with host preferences and some geographical restriction of disease.

The discrimination of molecular types is useful, but in some cases too limited for one to gain a more accurate understanding of the molecular epidemiology in any given region. To fully appreciate and track outbreaks and disparate cases in regions, a more rigorous molecular examination of isolates is necessary. This type of comprehensive analysis often reveals specific genotypes that are associated with clinical and veterinary cases. Several genotypes have been identified through molecular approaches including Amplified Fragment Length Polymorphism analysis (AFLP), Random Amplification of Polymorphic DNA (RAPD), and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis [16,41]. These molecular approaches are a rapid way to identify both the molecular type and common genotypes, but with the increasing discriminatory power of direct DNA sequence analysis and the continued reduction in costs, sequence analysis for molecular epidemiology is becoming increasingly applied to the analysis of *C. neoformans* and *C. gattii* strains [17,20,23,47,48].

The most widely utilized sequence-based typing method for the analysis of *C. gattii* molecular epidemiology is multilocus sequence typing (MLST, i.e., molecular bar-coding) [49]. This method is robust as it is portable between labs and allows for a simple deposition of sequence alleles into public sequence-repository databases. Most often, discriminatory sequences based on unlinked genomic loci are employed for the analysis of isolates [17,47,48].MLST revealed that the four molecular types exhibit no examples of nuclear allelic exchange in >300 global isolates, supporting the hypothesis that they are cryptic species [17,20]. Furthermore, MLST analysis of a large number of global isolates showed genotypic diversity within each molecular type, often associated with specific geographic regions, such as a high prevalence of VGI in Australia and common VGIV genotypes restricted to sub-Saharan Africa [17,20,25].

One important application of molecular epidemiology was applied with the emergence of the Vancouver Island outbreak in 1999 and its subsequent expansion throughout the North American Pacific Northwest. First AFLP, then MLST, confirmed that there are two genotypes largely responsible for the outbreak, VGIIa/major and VGIIb/minor [22,41].

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Subsequent studies on the outbreak were then conducted to confirm these genotypes in the region and also to document that the outbreak had expanded into the United States [17,31,50]. Additionally, sequence-based analysis revealed that a third outbreak genotype, unique to Oregon (VGIIc/novel), is also contributing to this ongoing outbreak [19,20,32]. The use of sequence typing and its role in understanding the Pacific Northwest outbreak will be detailed further in the section focused on this outbreak.

MLST analysis is also useful in identifying fungal infection cases as a result of travel. A significant finding of the Pacific Northwest outbreak has been that the VGIIa/major genotype has yet to be found elsewhere globally in the environment or clinically. This observation was then useful when cases from patients with a travel history to the endemic Vancouver Island region began to appear with travel-associated VGIIa/major genotype infections now documented in patients from Alberta (Canada), Denmark, the Netherlands, and Switzerland [51–53]. There has also recently been a report of a patient in Japan infected with the VGIIa/major genotype (identical at 11/11 MLST markers examined), but further analysis will be necessary to determine if this is a true non travel-associated VGIIa/major infection or a related but distinguishable genotype as is known to occur elsewhere globally [20,54]. Unrelated to the outbreak was also the first reported case of *C. gattii* infection from the Southeastern United States [21]. In this case, the isolate was determined to be molecular type VGI (and distinct from VGII and rare VGI isolates in the Pacific Northwest) based on MLST analysis. This patient had traveled to central California, in which the VGI molecular type is known to be endemic.

While the analysis of coding regions is highly useful for discriminating genotypes of pathogenic fungi, in some cases molecular epidemiology aspects cannot be resolved in these highly clonal populations. This is especially evident from studies of the VGI and VGII molecular types of *C. gattii*, in which isolates are often indistinguishable based on MLST analysis alone. To increase the resolution of typing, several approaches have been employed to generate microsatellite-based markers, including Variable Number of Tandem Repeat (VNTR) markers. The initial study to employ this approach in *C. gattii* was focused on largely clonal *C. gattii* isolates from Australia and the United States that were VGI molecular type [21]. In this case, MLST analysis of an isolate from a North Carolina patient provided initial evidence that it was indistinguishable from clinical isolates from Australia and California and environmental isolates from Australia; however, after the addition of microsatellite markers, the environmental isolates were distinguishable from other clinical isolates based on both size variation in microsatellite PCR products and sequence differences in the VNTR markers [21].

The large clonal outbreak of *C. gattii* continues in the North American Pacific Northwest. As the isolates from the three outbreak genotypes (VGIIa/major, VGIIb/minor, VGIIc/novel) are highly clonal, the sequence-based VNTR approach was applied so that a combination of repeat variations and single nucleotide substitutions, insertions, or deletions could be assessed [20]. Overall, this approach demonstrated that there was some underlying diversity in the VGIIa/major genotype, particularly among three isolates from Oregon and several associated isolates from California that are closely related but not indistinguishable from the VGIIa major genotype based on SNPs in variable regions of the genome. Several of the distinguishing SNPs are related to similar alleles seen elsewhere globally in divergent VGII strains. However, these differences occur in non-coding regions and are therefore likely related due to homoplasy (i.e., similarity in isolates of different ancestry and therefore of independent origin and not identical by descent) and not shared ancestry. The results also showed that the groups of VGIIb/minor and VGIIc isolates examined were indistinguishable from each other (all VGIIc originated from Oregon), although a report of a VGIIc isolate from a patient in Idaho with travel history to Oregon showed a single base pair difference at

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an MLST locus, indicating a likely recent divergence/drift due to expansion of this genotype [32]. Overall, the application of the VNTR sequence-based method proved highly discriminatory to differentiate *C. gattii* isolates that otherwise appeared indistinguishable at the previous loci examined. These studies also aided in expanding our knowledge of the overall structure of global *C. gattii* genotypes.

3. Global overview of clinical and environmental populations and insights into the origins of the pathogenic *Cryptococcus* species complex

3.1. Global overview of clinical and environmental populations

Molecular typing is a method often utilized to study pathogenic microbes for purposes of outbreak analyses. In addition, the analyses of conserved non-repetitive sequences are the fundamental basis for population studies. Unlike SNPs, repetitive sequences are often subject to homoplasy and thus avoided, although these markers can be of use for non-phylogenetic clustering analysis, which is often insightful for studies of global population structures (see Section 2). For these reasons, studies often include only unlinked conserved unique sequences (predominantly coding) for phylogenetic approaches and population genetic analysis and in some cases combine non-coding variable markers for large clustering approaches. In this section we review approaches that have been employed to analyze the population genetics of *C. gattii*, including approaches to identify evidence for same- and opposite-sex mating, global and regional population studies, and overall trends in global populations, both environmentally and clinically.

While *C. gattii* is often studied due to its importance as a mammalian pathogen, its common ecological niche is in the environment. For this reason, and to also understand the roles of mating and recombination (both of which can lead to novel and pathogenic genotypes), studies of naturally occurring isolates and their population structures are of fundamental importance. Several landmark studies of *C. gattii* in the environment have been conducted in Australia. Studies focused on environmental, clinical, and veterinary isolates have been approached from a population perspective. Studies during 2003 of *Eucalyptus* tree isolates yielded results consistent with only clonality and no signs of sexual recombination [55]. However, more recent studies analyzing populations collected from individual *Eucalyptus* trees showed evidence for both same- and opposite-sex mating [56]. Taken together, this suggests that while clonal reproduction may be a common means of reproduction for this fungus, both same- and opposite-sex mating are occurring in the environment, perhaps in highly restricted local environments that in some cases may even be as restricted as a single colonized tree. Furthermore, studies analyzing the population structures of clinical and veterinary isolates in Australia found evidence for clonality in some populations, and a combination of clonality and sexual reproduction in others [57]. Supporting studies showed that these isolates retain their ability to sexually reproduce in laboratory-based mating assays [58]. Population-based studies in Australia provide a foundation for studies in other regions, which also often yielded results suggesting both clonality and recombination in local and global based analyses, with further additional evidence for laboratory-based mating supporting these results [17,20,22,45,59]. Studies examining both small and geographically dispersed isolates from the environment and infected hosts are fundamental to elucidating population structures, speciation dynamics, and evidence for same- and opposite-sex mating in naturally occurring populations.

3.2. Insights into the origins of the pathogenic *Cryptococcus* species complex

In addition to population studies, comparative genetic and ecological studies of both the pathogenic species of focus and related non-pathogenic species can yield insights into pathogen emergence. The origin and evolution of pathogens remain central questions in

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studies of both plant and animal diseases. One method to examine the likely origins of pathogens is to phylogenetically place the species into the context of closely related relatives, which in the case of *C. neoformans* and *C. gattii* are saprobic. The closest related species to the pathogenic *Cryptococcus* species are associated with insects or insect frass. Although these sibling species are often less studied than their medically relevant counterparts, they can offer significant insights into the evolution of the animal pathogens and how these pathogenic species might have arisen from insect-associated saprophytes.

Phylogenetic analyses indicate that the *Cryptococcus* species complex likely arose from the *Tremella* lineage, clustered closely with the Tremellales, Trichosporonales, Filobasidiales, and the Cystofilobasidiales [60–62]. Several of the species within these lineages are saprophytes that are commonly associated with insect debris, leading to the hypothesis that the pathogens emerged from an association within this environmental niche [63]. In support of this hypothesis, *C. gattii* has been isolated from both insect frass and wasp nests and *C. neoformans* has been isolated from honeybee hives, indicating that these animal pathogens may still in some cases act as an insect-associated saprophyte or pathogen in the environment [61,64,65]. While the evolutionary factors influencing the manifestations of mammalian pathogens from saprobes are unclear, the support for the hypothesis of emergence based on phylogenetic and ecological studies provides insights into the evolutionary history of the pathogenic *Cryptococcus* species complex.

In addition to the ecologic studies mentioned above, interactions between *Cryptococcus* and insects have been further studied by the development of a well-validated insect model of pathogenesis in the heterologous insect host *Galleria mellonella* [66–68]. Outcomes from this yeast–insect system have been shown to correlate with the murine model of infection. In fact, studies examining both *C. neoformans* and *C. gattii* wild-type and mutant isolates show similar mortality/attenuation properties compared to the mammalian infection model [21,63,69]. Additionally, the pathogenic *Cryptococcus* species were shown to be more virulent than their non-pathogenic insect-associated relatives [63]. This invertebrate model of infection is inexpensive and poses fewer ethical issues, allowing for more facile, high throughput analyses of the cryptococcal virulence composite.

4. Environmental aspects of *C. gattii*

The ubiquity of *Cryptococcus neoformans* in the environment has been described as the inevitable consequence of the association with pigeons and the admixture of bird guano, cryptococci, and soil [70]. In the case of *C. neoformans*, it was the increase in the number of susceptible hosts with the advent of the HIV/AIDS epidemic that resulted in the clinical appreciation of the relationship of environmental exposure to acquisition of disease. Compare this then to the relative obscurity of disease caused by *C. gattii*, seemingly geographically restricted based on early studies of stored clinical isolates [12]. In 1987, David Ellis, challenged by the lack of an identified environmental source of *C. neoformans* var. *gattii* to account for the excess cases of gattii-associated disease in Northern Australia, began to search for the ecological niche of this organism [71]. In succeeding years, Ellis and Pfeiffer described the successful isolation of *C. gattii* from *Eucalyptus* trees in Australia and California [72–74]. The rarity of gattii-associated disease in geographic locations that did not support the importation or growth of cold-intolerant Eucalypts fit this segregation theory, despite the lack of evidence of an environmental source in areas with both Eucalypts and gattii-associated disease such as Papua New Guinea [27,75,76]. In North America, it was unusual for clinical laboratories to differentiate pathogenic cryptococci and all were assumed to be *C. neoformans*.

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In 2001, however, a veterinary pathologist was the first to notice an increasing number of cases of cryptococcosis, all originating from a large island west of mainland British Columbia. Vancouver Island has a population representing only approximately 17% of the entire province. Human cases were also starting to appear, also disproportionately represented from the smaller population pool. The cases were characterized as not associated with HIV/AIDS and the ensuing emergence, described in animals by Stephen et al. [33] and reviewed in humans by Galanis and MacDougall [29], continues to the present day. The subsequent discovery of cryptococcal disease in Washington and Oregon are described in detail in other sections of this review [19,20,23,24,31,50,51,53,77,78].

Investigation of trees, soil, air, and water in the vicinity of animal and human cases resident on Vancouver Island resulted in the isolation of *C. gattii* major (VGIIa) and minor (VGIIb) genotypes co-existing within the same ecologic niche [17,22,41]. In contrast to the Australian experience, where the majority of cases of gattii-cryptococcosis were in aboriginal people living in sparsely populated areas, the British Columbia cases were predominantly from urban (e.g., Victoria is the provincial capital of BC) or scenic suburban communities popular with retirees due to the mild climate [71]. Airborne *C. gattii* propagules are present in endemic areas of BC in highest concentration during the dry summer months (April through October, peaking in July) [59]. The seasonality of airborne propagules is not associated with the flowering or production of pollen of any of the trees found colonized with the pathogen, nor in the diagnosis of the disease as the time of onset of symptoms varies considerably as described by MacDougall and Fyfe [79]. Duncan et al. [80] found an epidemiological association between logging activities or soil disturbance within 10 km of the residences of veterinary cases of gattii-cryptococcosis. Environmental investigation within the 10 km radius of cases has been fruitful in locating trees and soil colonized with *C. gattii*. These investigations have further elucidated areas that are permanently colonized, yielding positive samples over the last decade of repeated sampling (53).

C. gattii colonization is not homogeneous throughout the eastern coast of Vancouver Island, nor is the relative proportion of the major to minor genotypes the same between permanently colonized areas. Recent analysis stratified by GIS location identified several sites where 100% of isolates belonged to the major genotype (VGIIa), compared to sites where three genotypes (VGIIa, 37%, VGIIb 23% and VGI (14%) are routinely co-isolated along with *C. neoformans* molecular types VNI and VNIII (Bartlett and Kidd, unpublished data). However, to date, VGIIc has not been identified on Vancouver Island (Byrnes, Hagen, Bartlett, Heitman, unpublished data).

Air and soil samples have yielded some interesting new information regarding the emergent *C. gattii*. Concentrations of 10^5 per gram soil are not unusual at some of the permanently colonized sites, with the highest concentrations often found outside the drip line of the tree. Of the Vancouver Island samples, *C. gattii* concentrations are negatively correlated with organic carbon content and soil moisture [59]. Using Andersen six-stage air impaction sampling, the size range of the airborne propagules was determined, with approximately 88% being in the size range 3.3 to >7 μm, and 12% smaller than 3.3 μm, which is the range considered to be small enough to be inhaled deep into the lung and the alveoli ([59]; Bartlett, unpublished data). Not surprisingly, disturbance of soil or the aerosolization of wood particulate during logging or limbing of infected trees increases the airborne concentration of *C. gattii* 10–100 fold ([59]; Bartlett and Hingston, unpublished data).

Early in the investigation of the environmental niche of *C. gattii*, speculation arose regarding the eventual spread of the pathogen. Using databases of the addresses of veterinary or human cases, and the GIS locations of positive environmental samples, ecological niche

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modeling was used to predict the eventual range of potential colonization to aid in planning the public health response to the emergence. The environmental predictors tested in the model included topographic, climatic, biogeoclimatic and soil data. The significant predictors were low lying elevations, daily January average temperatures above freezing, and presence within the Coastal Douglas-fir and Coastal Western Hemlock xeric maritime biogeoclimatic zones [81]. These same conditions are present on the southwestern coast of British Columbia and further south into Puget Sound. It was not a surprise, therefore, when the first mainland cases without a travel history to Vancouver Island were identified, first in veterinary cases, subsequently in humans in British Columbia [23], Washington [50], and Oregon [19,20,23,31,78]. In corroboration, positive environmental samples have been collected from air, soil and wood from mainland BC and Washington.

Much discussion has resulted from theories regarding how and when *C. gattii* came to BC. Of interest is a clinical culture isolated in Seattle (NIH444) and deposited in the American Type Culture Collection by June Kwon-Chung in the early 1970s that is indistinguishable from the major genotype, VGIIa. Duncan et al. [34] looked for potential wildlife reservoirs, but isolation of *C. gattii* from feral birds and mammals has been sporadic and not predictive. In the case of marine mammals, the travel path of the host was unknown prior to washing ashore with fatal cryptococcosis [33,37]. Anecdotally, *C. gattii* was recovered from the feces of two psittacine birds being treated for cryptococcomas, but the role that the fecal material of caged birds has in spreading *C. gattii* into the environment is unknown (Bartlett, unpublished data). To date, none of the cold-tolerant eucalyptus tree species surveyed in BC or Washington have been positive for *C. gattii*.

In summary, the emergence of *C. gattii* in the Pacific Northwest was unexpected, but the knowledge gained has been a unique example of the need for an interdisciplinary approach to craft the public health response. Early in the emergence, information was rapidly disseminated to primary care physicians and veterinarians in British Columbia. Public media coverage was less helpful in conveying accurate information [82]. The economic consequences have yet to be tallied, and include the cost of hospitalizations, antifungal medications, and loss of tourism to the area [83]. The value to understanding this novel pathogen, however, has been enormous, as evidenced by the speed with which the genome sequencing has been undertaken by an international consortium of researchers [84]. There is still much to be learned about the complex interactions between the environment, microbial ecology, and the ramifications for human health from present and future fungal pathogens.

5. Clinical aspects: human and veterinary infections

Cryptococcosis, the manifestation of infections caused by the pathogenic *Cryptococcus* species, is a severe and often life-threatening disease in humans and myriad wild, agrarian, and domestic mammals. Furthermore, infections can occur in both terrestrial and marine mammals, highlighting the ubiquitous nature of this pathogen in a wide range of terrestrial and aquatic environments. These infections are also complex clinically, as they commonly result in pneumonia, meningitis, and cryptococcoma formations in humans, and additionally may present as skin infections, particularly in feline and canine cases but also in humans [11,21,34–37,80,85–91]. The clinical course of infections are complex, and treatments are challenging, often long-term, and costly. These issues become particularly apparent in developing regions of the world where access to more effective therapeutic strategies is frequently limited and maintaining aggressive treatments for extended periods of time is not feasible.

Along with the differences in ecological and epidemiological features between *C. gatti* and *C. neoformans*, there has been a history of attempts to distinguish their clinical presentations

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and outcomes [92]. There are two large reviews of *C. gattii* compared to *C. neoformans* prior to the recent Vancouver Island outbreak and generally these comparisons came to the same clinical conclusions [93,94]. First, *C. gattii* occurs by percentage significantly more common in immunocompetent patients than *C. neoformans*. Second, *C. gattii* frequently produces disease with focal cryptococcomas in the brain or lung. These mass lesions are associated with either CNS complications such as hydrocephalus, cranial nerve palsies, and seizures or large inflammatory lesions in the lung that either require prolonged therapy and/or surgical intervention. Third, there are some suggestions by clinical experience that *C. gattii* requires more prolonged antifungal therapy and more commonly surgical removal of cryptococcomas. In fact, there remain inconsistent data on the in vitro susceptibility of *C. gattii* strains. For instance, there are both studies that support a similar MIC range to azoles as *C. neoformans* [95,96] and some recent data from the Vancouver Island outbreak strains that suggest some of the strains/genotypes possess increased resistance (West and Marr, unpublished observations). It may be that genetic subtypes vary in their susceptibility [97]. It is also likely that the majority of the clinical resistance for *C. gattii* disease is influenced by host factors such as immune reconstitution syndrome (IRIS) and with primarily immunocompetent patients the actual outcome of these difficult to manage infections is better than *C. neoformans.* In HIV-infected patients, it appears that *C. neoformans* and *C. gattii* have the same outcome in one center [98]. Specifically, in the Pacific NW outbreak with these new *C. gattii* strains there has been less mortality in Canada compared to United States [29,99]. The reason for this is unclear and some of it may still be related to a smaller sample size within the United States. After review of present data on outcomes, in vitro drug susceptibility and clinical experience, the 2010 IDSA guidelines have attempted to reflect the present knowledge base and that is to treat *C. gattii* similar to *C. neoformans* [30]. However, there was mention of how *C. gattii* may challenge the clinician in its management of cryptococcomas (brain and lung), chronic conditions of hydrocephalus, and at times slow improvement in symptoms on standard antifungal regimens.

Although the clinical manifestations may overlap between *C. gattii* and *C. neoformans*, it has been proposed that *C. gattii* infections and disease may more commonly present during the initial (acute) infection rather than present with reactivation disease [100]. This pathogenetic understanding is based on two indirect observations. First, serological studies in children show that they commonly have antibodies to *C. neoformans* but rarely *C. gattii* and second, *C. neoformans* commonly presents as the immunosuppressed patient's immunity wanes and much less of *C. gattii* disease occurs in immunosuppressed patients. However, it is important to emphasize that this impression is not absolute. It is also noted that serologies may be influenced by epidemiologic exposures and specific risk factors to the fungus. Furthermore, reactivations in some cases of *C. gattii* are supported by transplant infections (occurrence in non-endemic locations after living in an endemic area) [101] (although the patient in this case did also have travel to Africa 6–7 months prior to disease presentation) or natural animal infections that appear to be recurrent with the same *C. gattii* strain [102]. Even the use of the reduced numbers of immunosuppressed patients with *C. gattii* is not a solid insight into pathogenesis since we are now observing more cases during HIV infection and even with the Northwest United States outbreak the number of *C. gattii* infections in immunosuppressed individuals is substantial. Therefore, although there is some suggestion that the pathophysiology of *C. gattii* and *C. neoformans* might be different this is uncertain to what extent and to its clinical relevance. A final point to the pathophysiology and clinical presentation between *C. gattii* and *C. neoformans* is that the species can be configured differently for virulence factors. For example, in the trehalose pathway that is essential for disease production, the pathway's genetic influence on virulence factors such as capsule formation and melanin production differ between *C. gattii* and *C. neoformans* [103,104]. Therefore, our present knowledge base has documented differences in virulence factors between species and strains and this may be important to outcome in individual

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patients. Furthermore, the Pacific NW outbreak has taught us that with its massive human exposure to *C. gattii* propagules, only a small fraction of exposed humans came down with disease and this demonstrates that the genetic susceptibility of the host remains a primary factor in what direction this infection will travel.

In the clinical setting, the current IDSA cryptococcal treatment guidelines outline similar treatment schedules for *C. gattii* and *C. neformans* because there are no definitive trials that prospectively compare regimens and outcomes between the two species [30]. However, the guidelines do acknowledge that special care should be used to examine for the more likely possibility of cryptococcoma formation in *C. gattii* cases. Also, care should be given to appreciating the immune status of the patient, as there are a higher percentage of immunocompetent patients with cryptococcal infections attributable to *C. gattii* [30]. Therefore, these patients may present with impressive inflammatory masses and immune reconstitution inflammatory syndrome may become a prominent feature during treatment course and thus require clinical attention. In another clinical concern, several studies have recently documented some azole resistance in *C. gattii*, particularly among some of the highly virulent molecular type VGII genotypes responsible for the Pacific Northwest outbreak [32,105]. Although whether *C. gattii* strains in general have higher minimum inhibitory concentrations (MICs) than *C. neoformans* strains remains uncertain, clearly the apparently clinical response to treatment suggests that *C. gattii* infections may appear somewhat resistant to many treatment strategies. The CDC recommends that amphotericin B or its lipid products should always be used as an induction therapy followed by fluconazole maintenance therapy for *C. gattii* for the Pacific Northwest outbreak strains, whereas in non-CNS *C. neoformans* cases fluconazole can still be used successfully (Harris et al. 48th IDSA poster presentation #642, [30]). When the patient's immune status, with its increased predilection for cryptococcoma formation and higher rates of azole resistance are considered together, it suggests that *C. gattii* infections may need to be treated more aggressively than other cryptococcal infections and this may be particularly true in the Pacific Northwest outbreak setting. Another clinical factor is that *C. gattii* infections without risk factor appearance (i.e., normal host) may often go undiagnosed for longer periods of time and not be appreciated by clinical providers. There is generally a lack of speciation between *C. gattii* and *C. neoformans* in clinical labs. Overall, *C. gattii* infections in humans are complex and clinical studies specifically addressing *C. gattii* prognosis and treatment in a prospective manner need to be conducted.

While a large focus of cryptococcal disease is on human infections, veterinary infections are also a significant cause of animal morbidity and mortality worldwide. Infections have been documented in a broad range of domestic, agrarian, and wild mammals, indicating that many mammals may be subject to disease and that cases in wild animals may frequently go unrecognized [19,31,33–37,80,85–87,89,91,106–108]. Infections in animals are of concern for economic factors (agrarian), quality of life for owners (domestic), and for wildlife concern particularly in the cases of koalas and marine mammals [19,20,33,37,77,78,91,109]. Furthermore, in cases of novel outbreaks (Vancouver Island outbreak and United States expansion), animal infections served as sentinel cases and helped highlight specific geographic expansion and emergence because many of the animals were non-migratory [21,33,34,78].

Cryptococcal infections in humans and other animals, particularly those caused by *C. gattii*, are not difficult to diagnose but the clinician must consider it in their differential diagnosis. The disease presentation can be diverse in humans and animals. Advances in early diagnosis and access to effecting treatments in humans and animals can decrease attributable morbidity and mortality; study of yeast strains with clinical information can help understand the evolution of virulence. In fact, the Vancouver Island outbreak represents an ideal

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opportunity to study the genetic host susceptibility to cryptococcal disease. Clearly, the island inhabitants (>700,000 individuals) had substantial exposure to the fungus but only a small fraction came down with disease. Genetic studies may identify find polymorphisms in innate immune genes in those with disease. Clinical and veterinary aspects also highlight the multidisciplinary and collaborative approach needed to address the many issues surrounding *C. gattii* infections.

6. The Pacific NW outbreak: review and outlook

As of 1999, *C. gattii* has emerged as a primary pathogen in North America, including both Canada and the United States [17,19,20,22–24,29,41,77]. This outbreak now spans a large geographic range, with levels of infection as high or higher than anywhere else globally. For instance, the annual incidence on Vancouver Island is approximately 25 cases/million people [29]. The only two reports with higher overall incidences are one examination of native Aboriginals in the Northern Territory of Australia, and a study conducted in the central province of Papua New Guinea [26,27,29]. The VGI and VGII molecular types are the primary ones associated with illness in otherwise healthy individuals [24]. Infections due to VGI have been reported at high rates among populations in Australia, while the levels of VGII infection are higher in the Pacific NW, where >95% of all cases are attributable to this molecular type [11,17,18,24,45]. The appearance of *C. gattii* in North America is startling because this is the first major emergence of this species in a temperate climate [59,79], and unlike *C. neoformans*, this pathogen had previously been geographically restricted to tropical and sub-tropical regions throughout the world [11,12,110,111]. To examine the evolutionary aspects of this unprecedented outbreak, efforts were undertaken to study the molecular epidemiology and characteristics of isolates collected from humans, animals, and the environment. These efforts have and will continue to shed light onto several key features of this outbreak, while other contributing factors remain elusive.

A central question from the analysis of the Vancouver Island outbreak relates to the origin of the first novel genotype observed as part of the outbreak, VGIIa/major. This genotype has been responsible for the vast majority of all infections reported in British Columbia [22,41]. As in the sibling species *C. neoformans*, many *C. gattii* populations are predominantly comprised of a mating type isolates, and to date all isolates related to the outbreak have been exclusively α. A seminal finding by Lin and colleagues in 2005 was the discovery that monokaryotic fruiting represents a novel mode of α–α unisexual reproduction (including meiosis) [112–114]. This finding, in combination with the discovery of an α/α VGIIa/major diploid isolate from Vancouver Island (RB59), and molecular comparisons between the VGIIa/major genotype and the less prevalent VGIIb/minor genotype that is also found in Australia led to the hypothesis that same-sex mating may have produced the virulent VGIIa genotype and is responsible for ongoing production of infectious spores. An alternative hypothesis is that opposite-sex mating, possibly in South America where *MAT*a isolates similar in genotype have been discovered, contributing to the origin of the outbreak isolate genotype [17,115]. In fact, the VGIIa/major subgroup has been shown to be more fertile in comparison to the VGIIb/minor subgroup [20,116]. Further studies of the global VGII population also suggest that recombination is still occurring [20]. While the origins of *C. gattii* VGIIa/major in North America remain uncertain, it is clear that this emerging pathogen has recently invaded the United States, and that in addition a new unique and highly virulent genotype in Oregon, VGIIc, has also arisen [19,20,31].

In response to this outbreak, a multidisciplinary *C. gattii* working group was established to address the epidemiology, clinical features, and basic science questions surrounding this outbreak [78]. The overall incidence remains low even in endemic areas and very little is currently known about how or why specific humans and animals develop disease. It

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probably involves unique host factors, including possible genetic predisposition in addition to steroid treatment and other identified predisposing risk factors [117]. In addition, the precise origins of the VGIIa/major and VGIIc/novel genotypes remain elusive: the VGIIa outbreak isolates are indistinguishable from isolate NIH444, indicating it has been circulating in the region for at least ~40 years; the VGIIb outbreak isolates are indistinguishable from fertile recombining Australian populations and therefore likely originated there; and VGIIc outbreak isolates are unique to Oregon so likely originate from an under-sampled global niche or arose locally. Substantial progress has been achieved in addressing the molecular epidemiology and expansion of the outbreak, and also the phenotypic characteristics that make these genotypes unique. However, many critical questions remain to be addressed in the future to understand the evolutionary dynamics of this unprecedented *C. gattii* emergence in North America. Expanded environmental sampling, further phenotypic characterization of associations with host animals and plants, and genome sequencing of *C. gattii* mitochondrial and nuclear genomes in addition to those available for the VGI isolate WM276 and the VGIIa/major outbreak isolate R265 [84] should be conducted. Furthermore, host genetic profiling is needed.

7. Conclusions

Until 1999, the vast majority of studies surrounding *Cryptococcus* pathogenesis focused on the globally distributed sibling species to *C. gattii*, *C. neoformans*. While the North American Pacific Northwest *C. gattii* outbreak has increased the profile of infections caused by the less-common species, we now also appreciate that *C. gattii* may be more frequently found in HIV/AIDS patients than previously estimated [46] (Byrnes et al, *PLoS Pathogens*, in revision), and that outside of the Pacific Northwest outbreak there may also be a number of attributable cases from any of the four molecular types in both otherwise healthy and immunosuppressed hosts [21,51–54,118].

Examination of isolates by high-resolution molecular and phenotypic analyses can facilitate in determining and understanding the dynamics of expansions into new regions, help to reveal the emergence of novel genotypes, and allows discrimination of cases not related to specific outbreak genotypes. Furthermore, molecular typing can support whether or not specific cases are likely travel-associated. Characterizing genotypes and molecular types also allows for the classification and phenotypic comparisons of virulence, highlighting as in the case of the Pacific Northwest that specific genotypes causing disease are highly virulent in animal models of infection. In-depth analysis of populations also allows for recombination studies, leading to a greater understanding of the roles that both same- and opposite-sex mating may play in the formation of infectious spores and the generation of genetic diversity and thus novel genotypes via meiotic events.

Recent lines of evidence suggest that mitochondrial inheritance and recombination could also play significant roles in the evolution and virulence composite of *C. gattii* [119–121]. Mitochondrial recombination or exchange requires cell–cell fusion, and it is hypothesized that this type of event could also lead to other nuclear or plasmid genetic exchange [122,123]. Future studies should also expand environmental sampling, apply further phenotypic characterizations of associations with host animals and plants, and utilize next generation sequencing to analyze more representative *C. gattii* mitochondrial and nuclear genomes. Additionally, epidemiological, clinical, and veterinary studies need to be continued, with an active multidisciplinary collaboration between all of those with vested interests in this area of study.

Why *C. gattii* emerged in a temperate climate for the first time remains unclear, and importantly it is not known how far the outbreak will expand and if new risk areas may

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occur in other temperate regions. Furthermore, if the outbreak will inexorably expand down the coast or alternatively discontinuously jump to new regions remains unclear. This punctuates the Pacific Northwest outbreak as a focal point for studies and a paradigm for novel emergent fungal diseases. To this end, it is critical that clinical laboratories and veterinary diagnostic laboratories begin to consider assigning species identity to clinical isolates, thereby distinguishing *C. neoformans* and *C. gattii*. In addition to advancing the field of *C. gattii* research, the reviewed studies also contribute to the ongoing research surrounding the dynamics of emerging and reemerging diseases and outbreak examinations. In host-microbe interactions one tenant is clear and well-described by Lewis Carroll's Red Queen adage: "It takes all the running you can do, to keep in the same place." As advances in treatment continue, microbes gain drug resistance/virulence attributes and new pathogens emerge, tipping the balance between the host and pathogen populations toward disease emergence. This co-evolution will undoubtedly continue, and further understanding of both the host and its pathogens are required so that advances in the prognosis, treatment, prevention, and eradication of infectious diseases can be achieved.

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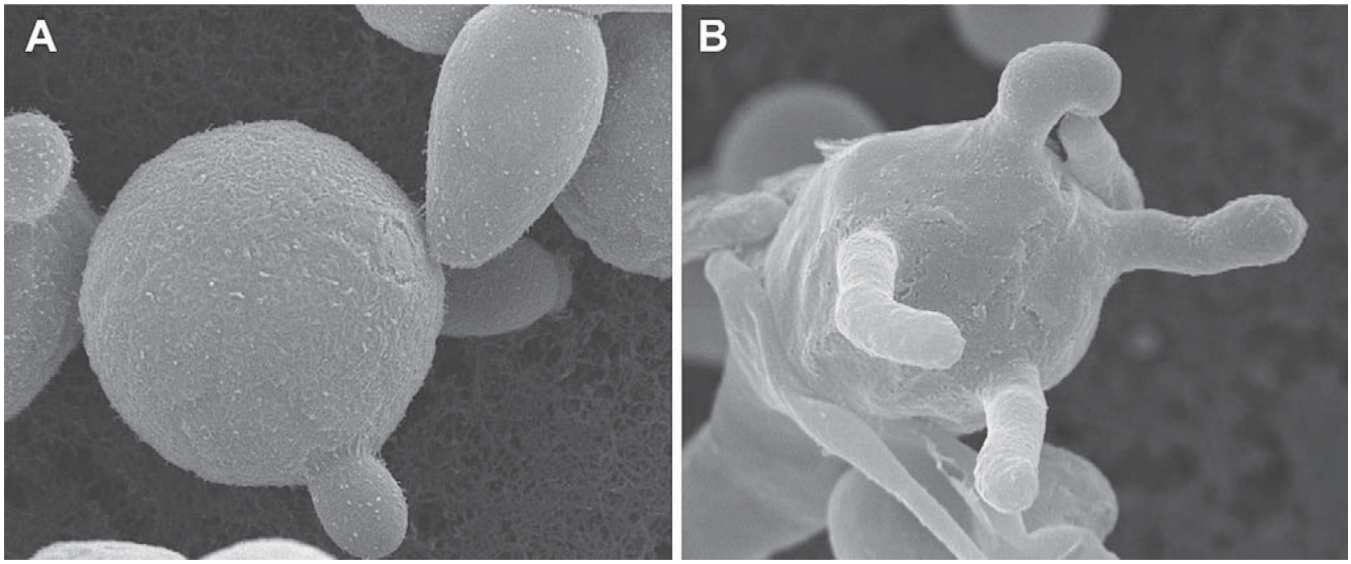

Fig. 1.
Morphology of *C. gattii*. Scanning electron microscopy illustrates *C. gattii* asexually producing as a budding yeast (A) and sexually reproducing to form a basidium with four emerging basidiospores (B).

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Table 1

Descriptions of the four *C. gattii* molecular types.

Molecular type	Clinical features	Environmental features	Distribution
VGI	Most common molecular type in humans and animals and highly clonal	Commonly associated with *Eucalyptus trees*, particularly in Australia [17,58]	Global with high distribution in Australia
VGII	Responsible for Pacific Northwest outbreak, clonal in outbreak region but diverse globally, highly virulent genotypes (VGIIa, VGIIc) identified	Associated with native tree species, with common isolation from Douglas-fir and Alder trees in British Columbia [59]	Global, also the cause of the first outbreak in a temperate climate on Vancouver Island
VGIII	Frequently associated with infection of HIV/AIDS patients	Isolates are highly fertile, and have been found in *Corymbia ficifolia* (Red Flowering Gum, Colombia) [124] and *Eucalyptus* (California) [22]	Global, high levels observed in Southern California, Mexico, and South America
VGIV	Frequently associated with infection of HIV/AIDS patients	Largely unknown, but one positive isolate from an Almond tree [43,125,126]	Rare, reported in Africa, India, and South America