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**Cryptococcosis in Southern-Brazil:
Molecular, antifungal and *in vitro*
cytokine induction studies**

Patricia Fernanda Herkert

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Molecular, antifungal and *in vitro*
cytokine induction studies**

The research presented in this thesis was performed at the Centre of Expertise in Mycology Radboudumc/CWZ, the Radboud Institute for Molecular Life Sciences in the Department of Medical Microbiology and at the Canisius-Wilhelmina Hospital in the Department of Medical Microbiology and Infectious Diseases, Nijmegen, The Netherlands.

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Cryptococcosis in Southern-Brazil: Molecular, antifungal and in vitro cytokine induction studies

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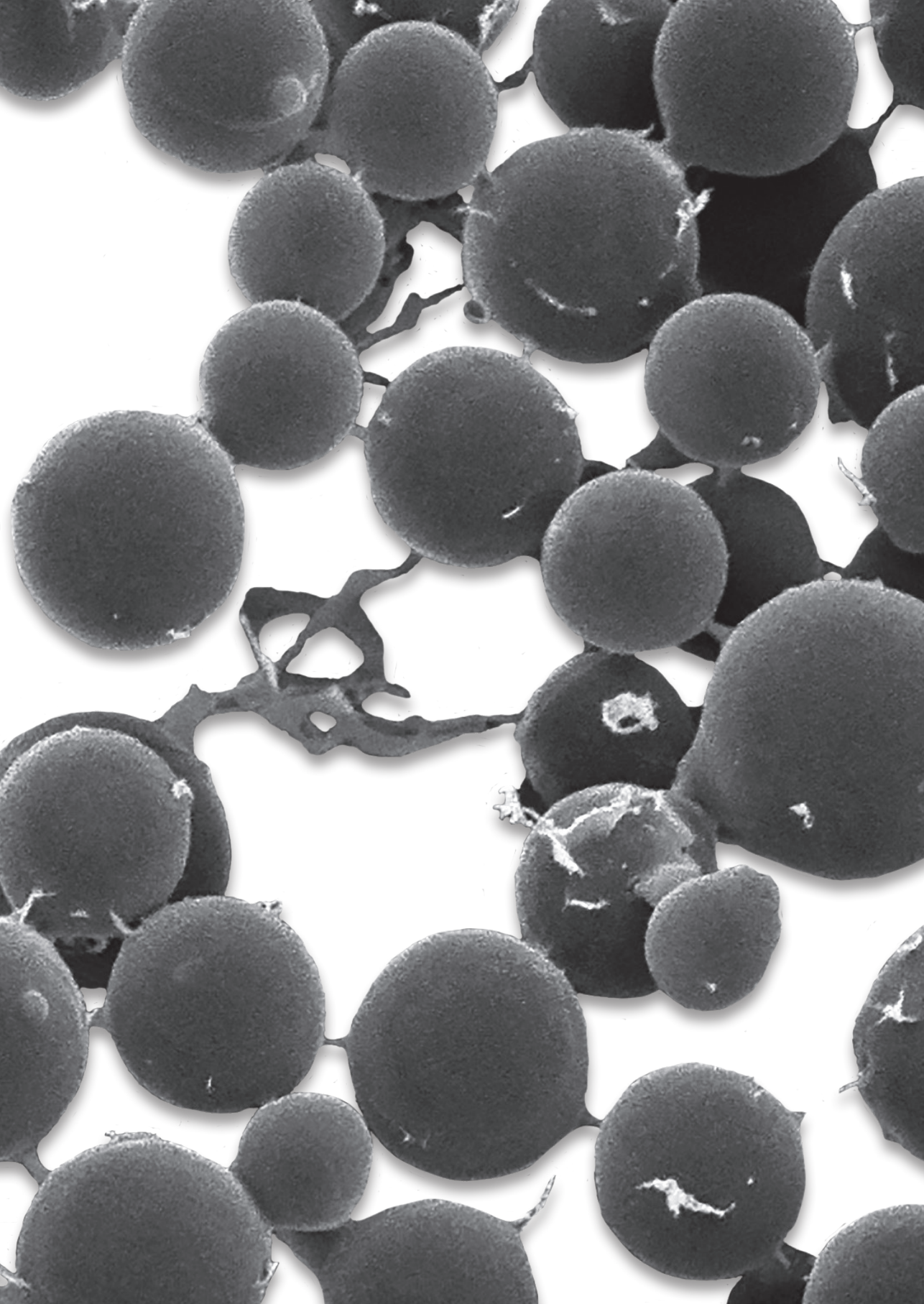
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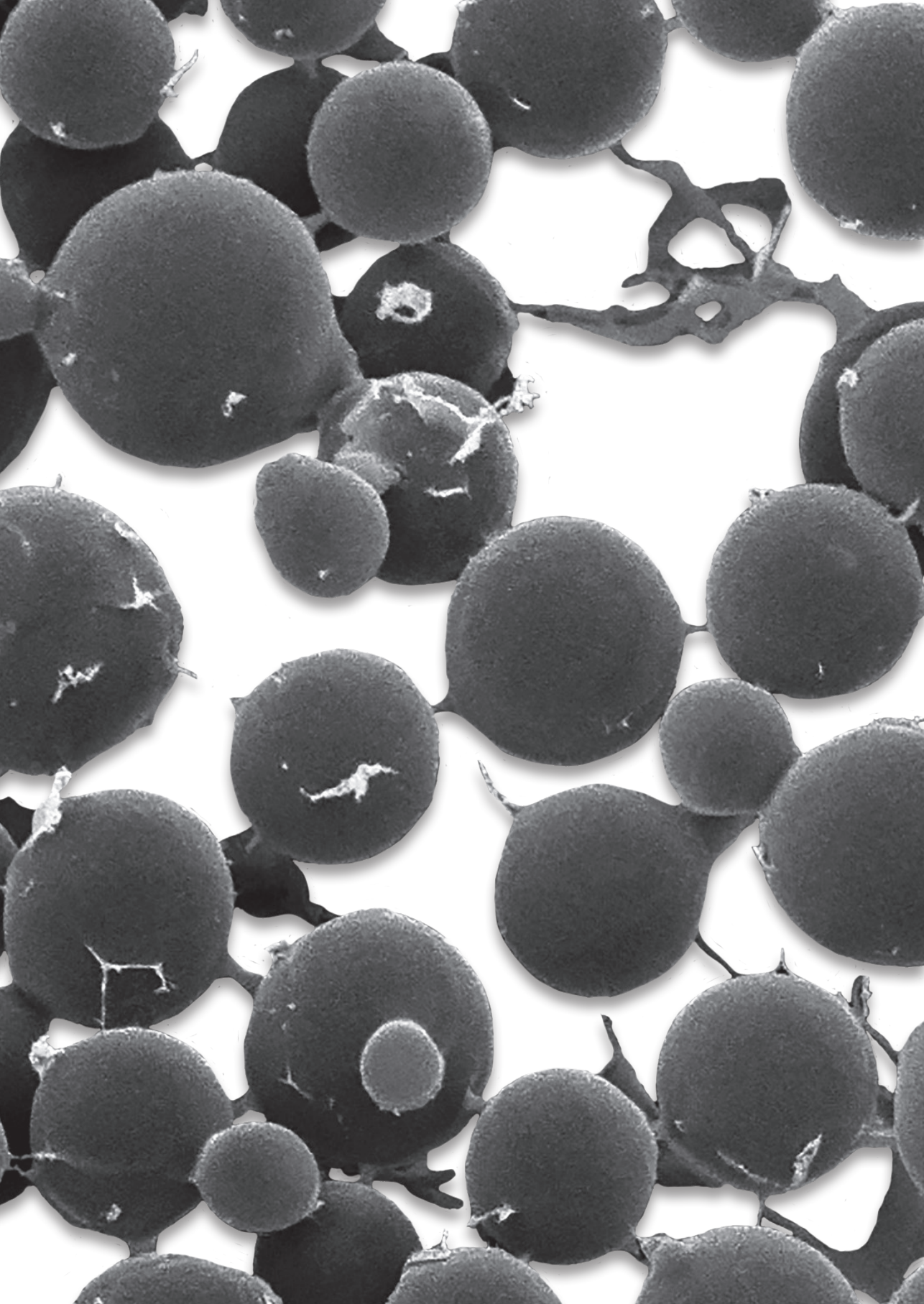
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Dedico esta tese aos meus pais, meus irmãos e ao meu noivo.

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Chapter 1

1

General introduction and outline of the thesis

Introduction

Cryptococcosis is a systemic infectious disease caused by the encapsulated yeast *Cryptococcus* spp., acquired by inhalation of basidiospores present in the environment [1]. Meningoencephalitis is the main clinical manifestation, with serious evolution [2] and related to neurological sequelae as blindness, deafness, muscle weakness and dysphagia [3]. Besides central nervous system (CNS) infection, pulmonary and skin infection may be observed [4, 5]. Annually, approximately 223.000 meningitis cases are reported in HIV patients around the world, with 180.000 deaths in 2014 [6].

Cryptococcus yeasts are present in the environment and are frequently recovered from soil, dust, trees and wood [7, 8]. The species of *C. neoformans* and *C. gattii* complexes are the main etiologic agents of cryptococcosis [9]. *Cryptococcus neoformans* species complex is cosmopolitan and is involved with the majority of cryptococcosis cases among immunocompromised patients around the world, mainly the ones who received organ transplantation, use corticosteroids or have AIDS [10]. *Cryptococcus gattii* species complex is emergent, initially found in tropical and subtropical regions with recent expansion to cold and temperate climates, as British Columbia in Canada, Washington and Oregon in the United States, where cryptococcosis outbreaks by *C. deuterogattii* (VGIIa/AFLP6A) were reported [11-13]. The environmental dispersion is believed to occur due to fungus transport by wood products, water, air, animals, insects and humans [14].

Cryptococcus taxonomy

Cryptococcus is a basidiomycetous yeast that belongs to the tremellomycetes class and has species in the Tremellales, Trichosporonales and Filobasidiales orders [15, 16]. *Cryptococcus* genus has more than 100 species, but the species inside the *C. neoformans* and *C. gattii* complexes are medically important because they are the main etiologic agent of human and veterinary cryptococcosis [9, 15-21]. *Cryptococcus* taxonomy was revised recently, and many species were transferred to other genera, for example, *C. laurentii* was transferred to the genus *Papilliotrema* [15, 16, 22]. *Cryptococcus neoformans* varieties raised to species: *C. neoformans* (formerly *C. neoformans* var. *grubii*/genotypes AFLP1/VNI, AFLP1A/VNII and AFLP1B/VNII) and *C. deneoformans* (formerly *C. neoformans* var. *neoformans*/genotype AFLP2/VNIV) [22]; and five genotypes of *C. gattii* species complex were raised to five species: *C. gattii sensu stricto* (AFLP4/VGI), *C. bacillisporus* (AFLP5/VGIII), *C. deuterogattii* (AFLP6/VGII), *C. tetragattii* (AFLP7/VGIV) and *C. decagattii* (AFLP10/VGIV) [22].

Although the taxonomic revision was established after 15 years of debate, part of the scientific community prefers to use only the term “species complex” [23] to refer to the genotypes/species belonging to the complexes, despite the evidence which support the revision [24]. This divergence was debated during the taxonomy session at the “10th International Conference on *Cryptococcus* and Cryptococcosis” in 2017 in Foz do Iguaçu, Brazil. During the session, arguments for and against the taxonomic review were presented and, during the discussion, there was broad support for taxonomic review. In the present work, the taxonomy suggested by Hagen et al. (2015) was used to quote *C. neoformans* and *C. gattii* species complexes [22].

***Cryptococcus* spp. environmental niches**

Environmental niches of *C. neoformans* and *C. gattii* has been widely investigated in the world, showing several environmental sources previously not considered for the presence of the fungus [25-28].

Cryptococcus neoformans s.s. (AFLP1/VNI) is the most prevalent species in the world and is responsible for the majority of cryptococcosis cases [29, 30]. However, *C. deneoformans* (AFLP2/VNIV) occurs more frequently in Europe and North America [30]. *Cryptococcus neoformans* species complex has been isolated mainly from pigeon droppings that are easily found in the urban perimeter [31-34], and from Passeriformes and Psittaciformes birds that live in domestic areas [35]. In addition, many tree species have been reported in the literature as environmental reservoirs of *Cryptococcus*, such as *Colophospermum mopane*, *Acacia* sp., *Ziziphus mucronata*, *Eucalyptus* sp., *Olea europaea*, *Cassia* sp., *Ficus* sp. and *Tipuana tipu* [34, 36-41].

The first environmental isolation of *C. gattii* s.l. were obtained from *E. camaldulensis* in Australia, indicating a specific association between *C. gattii* s.l. and *E. camaldulensis* in this region. According to Ellis and Pfeiffer (1990) the dispersion of *C. gattii* s.l. appeared to occur in late spring when the flowering period of *E. camaldulensis* started [42]. In addition, the distribution of *E. camaldulensis* corresponded to the region of endemic occurrence of cryptococcosis caused by *C. gattii* s.l. in Australia [42]. In other regions, *C. gattii* species complex has been isolated from a wide variety of trees, such as *Acacia xanthophloea*, *Ceratonia siliqua*, *Corymbia ficifolia*, *Moquilea tomentosa* and *Pseudotsuga menziesii* [27, 40, 43-45]. However, *C. deuterogattii* has also been recovered from wood houses' dust [8] and *C. gattii* s.s. from avian droppings [35, 40].

Despite all studies in *Cryptococcus* spp. and cryptococcosis, the environmental reservoir and species diversity are unknown in many countries. The knowledge

of local epidemiology is important to recognize possible sources of human infection, as well as the most frequent species, and to trace the best measures of cryptococcosis prevention and control.

Virulence factors

Cryptococcus neoformans and *C. gattii* have the ability to cause infection in immunocompromised and immunocompetent people [9]. The size of infecting cells (1.8 – 3 μm) allows pulmonary alveoli invasion and arrest in brain capillary [1, 46, 47] and thermotolerance enables cell multiplication inside the host [48]. Protease and phospholipase enzymes are important for tissue invasion [49] and melanin production helps the fungus against the oxidative stress produced by host phagocytic cells [49]. As well as, superoxide dismutase is involved with resistance to oxygen radical-mediated damage addressed to cryptococcal cells inside macrophages [50].

Cryptococcus neoformans is able to proliferate inside macrophage's phagosome [51, 52] and through this interaction cryptococcal cells may disseminate to other tissues, such as CNS, via Trojan horse mechanism [53, 54]. In addition to reach the CNS, paracytosis is used to cross tight junctions of brain endothelium [55] and transcytosis to move directly through the endothelial layer [56]. Urease facilitates cryptococcal transmigration by promoting damage to the microcapillary cells mediated by ammonia production, this increases the permeability of the endothelium and allows the dissemination to the brain [47, 57].

The polysaccharide capsule, composed of glucuronoxylomannan and galactoxylomannan, is an important virulence factor of cryptococcal cells involved in avoidance of phagocytosis. According to Bojarczuk and colleagues (2016), capsule size is a limiting factor of phagocytosis by macrophages that uptake more efficiently cryptococcal cells with small capsule size [58]. The cell size is another limiting factor, host mononuclear cells are unable to phagocytose titan cells because their giant size [59].

Immune response against *Cryptococcus* spp.

Cryptococcal mannose and beta glucan are the primary pathogen recognition motifs and are determinants in early interactions in the lung [60]. In addition, cryptococcal capsule accumulates C3 that facilitate phagocytosis [61, 62] by alveolar macrophages and dendritic cells in lungs [63]. Degraded cryptococcal components are loaded by major histocompatibility complex class II leading to adaptive immune system activation [64]. Th1 and Th17 profiles activate macrophages anti

cryptococcal activities associated with intracellular cryptococcal clearance [60, 65]. However, cryptococcal capsule polysaccharides induce a Th2 profile leading to cryptococcal intracellular growth, high levels of anti-inflammatory cytokines and low levels of proinflammatory cytokines [52, 66]. Interestingly, Garro and colleagues (2010) showed that eosinophils phagocytose cryptococcal cells and drive to a Th1 profile in rat cryptococcosis model and promote clearance of fungi [67].

Most studies drive to elucidate host immune responses during cryptococcosis are performed with human and animal cells stimulated with *C. neoformans s.l.*, but recently the immune response against *C. gattii s.l.* has been investigated. Based on this, it is known that *C. deuterogattii* polysaccharide capsule blocks the recognition of surface antigens by dendritic cells and despite be internalized and killed by dendritic cells, the fungus evades maturation of this phagocytic cells resulting in no increase of TNF- α and suboptimal T-cell responses [68, 69]. According to Angkasekwinai and colleagues (2014), *C. gattii s.l.* infection compromises Th1 and Th17 differentiation and downregulates chemokine expression (IL-12 and IL-23), leading to inadequate protective immunity [70].

Antifungal susceptibility

Different in vitro antifungal susceptibility was found among *Cryptococcus* spp. Overall, *C. gattii* species complex have higher minimum inhibitory concentration (MIC) to azoles than *C. neoformans* species complex [39, 71, 72], this was also observed for amphotericin B and flucytosine [73]. In Brazil, *C. deuterogattii* (AFLP6/VGII) clinical isolates showed high MIC values of flucytosine [74, 75] and fluconazole compared to *C. neoformans s.s.* (AFLP1/VNI) [74]. In addition, species inside the *C. gattii* species complex have different in vitro susceptibility. *Cryptococcus deuterogattii* (AFLP6/VGII) has high geometric mean MICs of flucytosine, fluconazole, voriconazole, itraconazole, posaconazole and isavuconazole when compared to *C. gattii s.s.* (AFLP4/VGI) [74, 76]. Lockhart and colleagues (2012) observed that *C. deuterogattii* (AFLP6/VGII) had high geometric mean MICs of fluconazole, followed by *C. bacillisporus* (AFLP5/VGIII), genotype VGIV (AFLP non-typed) and *C. gattii s.s.* (AFLP4/VGI) [77]. Despite the differences in antifungal susceptibilities among species, the initial therapy of cryptococcosis is chosen according to clinical manifestation and patient immune status [78].

Heteroresistance to antifungals is also observed among *C. neoformans* and *C. gattii* species complex when high concentration of itraconazole and fluconazole are tested [79, 80]. This phenomenon is related to phenotypic changes of cryptococcal cells as variation in cell and capsule size, decrease of ergosterol in the

cell wall, less susceptibility to oxidative stress and higher capability to proliferate inside macrophages [80]. These traits may be related to failure and relapses, but the clinical importance of the phenomenon is not elucidated [79-81].

Antifungal susceptibility tests are useful to investigate failure and relapses after therapy observed in patients treated with amphotericin B and azoles, but few data in the literature correlate clinical outcome with antifungal susceptibility [82]. Besides most *Cryptococcus* spp. isolates are susceptible to polyenes, flucytosine and azoles [75, 83], the prolonged use of fluconazole during meningitis maintenance therapies in HIV patients may select non-wild type isolates to this antifungal [84].

Cryptococcosis

The most frequent clinical manifestation of cryptococcosis is meningoencephalitis, which consists of subarachnoid space infection with involvement of cerebral parenchyma. The patients present nonspecific clinical features such as headache, fever, malaise, increased intracranial pressure and altered mental status [85]. Other signs are meningism, papillary edema, cranial nerve palsy, neurological deficit, depressed level of consciousness, cognitive impairment and gait ataxia due to obstructive hydrocephalus with ventricular dilatation [85].

Pulmonary cryptococcosis rarely develops as an acute process with clinical manifestations similar to other pulmonary diseases. Patients present weight loss, prolonged fever, anorexia, fatigue, cough, mucus or mucopurulent expectoration and hemoptysis [86]. Dyspnea and pleural pain may appear less frequently. Imaging exams show unilateral infiltrates, miliary micronodules, dense spherical lesions (cryptococcomas), and unilateral pleural effusions [2, 86].

Cutaneous lesions may be observed in patients with AIDS or with organ transplants, disseminated cryptococcosis, sarcoidosis, or who are receiving corticosteroids [2]. The initial lesion usually presents as a painless, ulcerative, acneiform or molluscum-like papule, with evolution to vegetative lesion. In patients with severe immunodeficiency, the lesions may be associated with vasculitis. The appearance of subcutaneous nodules is more common in patients who received high doses of corticosteroids [2].

In immunocompetent hosts, primary lung infection is often asymptomatic and controlled, remaining dormant. However, when the host's immune system becomes compromised the infection can reactivate and then reach extra pulmonary sites such as the CNS [87].

Outline of this thesis

The aims of this thesis were to investigate the molecular population structure of clinical and environmental *Cryptococcus* spp. isolates, to determine epidemiological and phylogenetic relationships, and test in vitro antifungal susceptibility. In addition, we investigated the in vitro cytokine induction by *C. gattii* species complex.

This thesis consists of two sections: Section I contains the chapters 2, 3 and 4 which focus on *C. gattii* species complex genetic diversity, global distribution and in vitro cytokine induction. Section II contains the chapter 5 on genetic diversity of *C. neoformans sensu stricto*.

Chapter 2 describes the ecoepidemiology of the *C. gattii* species complex in developing countries from different continents. Besides that, we discuss some aspects of *C. gattii* species complex antifungal susceptibility and bring insights on the revised *Cryptococcus* taxonomy.

Chapter 3 describes the molecular characterization of *C. deuteroformans* clinical isolates by amplified fragment length polymorphism (AFLP), mating-typing and multi-locus sequencing (MLST), and the characterization of in vitro antifungal susceptibility to amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole and flucytosine.

Chapter 4 describes a comparison of *C. gattii* species complex ability to induce in vitro cytokines and antimicrobial molecules in human peripheral blood mononuclear cells (PBMCs). For this study we used reference strains of the *C. gattii* species complex.

Chapter 5 describes the distribution of genotypes of clinical and environmental isolates of *C. neoformans sensu stricto* as well as the in vitro susceptibility of the most frequently used antifungal agents for the treatment of cryptococcosis. For this study, 219 Brazilian isolates of *C. neoformans s.s.* were characterized for mating-type, serotype, AFLP, microsatellite pattern and antifungal susceptibility.

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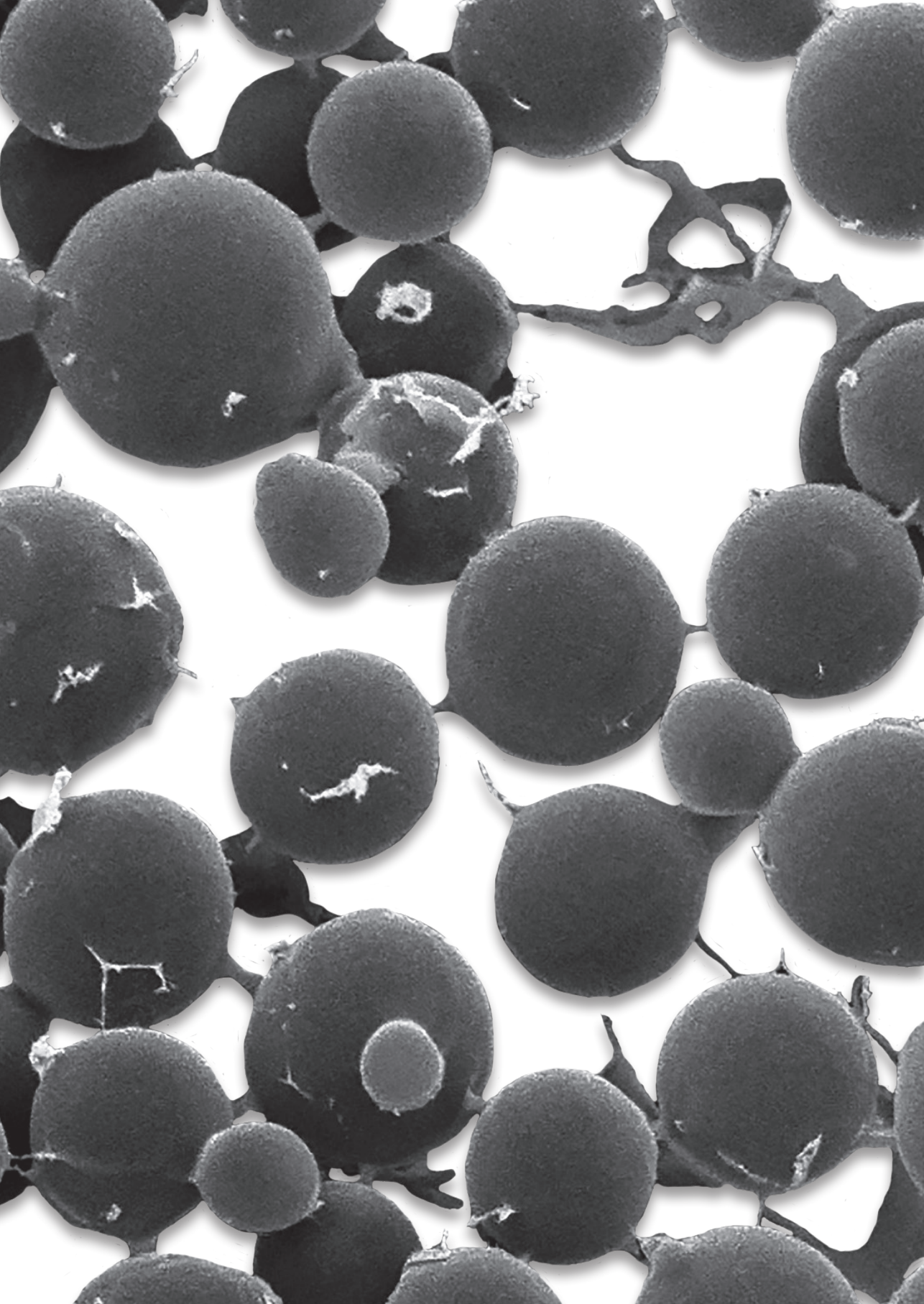
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Chapter 2

2

Ecoepidemiology of *Cryptococcus gattii* in developing countries

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Abstract

Cryptococcosis is a systemic infection caused by species of the encapsulated yeast *Cryptococcus*. The disease may occur in immunocompromised and immunocompetent hosts and is acquired by the inhalation of infectious propagules present in the environment. *Cryptococcus* is distributed in a plethora of ecological niches, such as soil, pigeon droppings, and tree hollows, and each year new reservoirs are discovered, which helps researchers to better understand the epidemiology of the disease. In this review, we describe the ecoepidemiology of the *C. gattii* species complex focusing on clinical cases and ecological reservoirs in developing countries from different continents. We also discuss some important aspects related to the antifungal susceptibility of different species within the *C. gattii* species complex and bring new insights on the revised *Cryptococcus* taxonomy.

Keywords: *Cryptococcus*; cryptococcosis; reservoirs; developing countries; ecoepidemiology

Introduction

Cryptococcosis is a systemic fungal disease caused by yeasts belonging to the *Cryptococcus neoformans/C. gattii* species complexes [1], affecting both immunocompetent and immunocompromised hosts and causing devastating diseases [2]. Cryptococcal meningitis is the most common mycosis associated with acquired immune deficiency syndrome (AIDS) patients with significant morbidity and mortality especially in sub-Saharan Africa, Asia, and Latin America [3]. It is estimated that approximately 225,000 new cryptococcal meningitis cases occur globally each year, the majority of which (73%) occur in sub-Saharan Africa [3].

Cryptococcus gattii sensu lato (s.l.) is an emerging pathogen, initially considered an endemic disease, affecting patients living in tropical and subtropical zones [4]. However, over time, the geographic distribution of *C. gattii s.l.* infections expanded to temperate climate regions including Canada and the USA [4–6]. In addition, many ecological niches have been investigated globally in an attempt to elucidate the environmental reservoirs [7–9].

In this review, we describe the ecological distribution of the *C. gattii* species complex and highlight the environmental reservoirs of this pathogen in developing countries. We also discuss some important points about the antifungal susceptibility of this species complex and changes in the *Cryptococcus* taxonomy that has recently been debated among researchers and clinicians.

***Cryptococcus gattii* species complex distribution in developing countries**

The *C. gattii* species complex was initially found in tropical and subtropical areas [10], but during the past two decades, the expansion to temperate climate regions was increasingly reported [5, 6, 11–15]. The ecological niches of the *C. gattii* species complex has been thoroughly investigated, and many global studies revealed that a plethora of tree species may be colonized by these pathogenic fungi [1, 7–9, 13, 14, 16–20]. The distribution of *C. gattii* species complex in developing countries is shown in the Figure 1 and Table 1. Based on these data, it became clear that the *C. gattii* species complex is not associated to a specific tree genus but that it has a predilection for plant/wood debris in general.

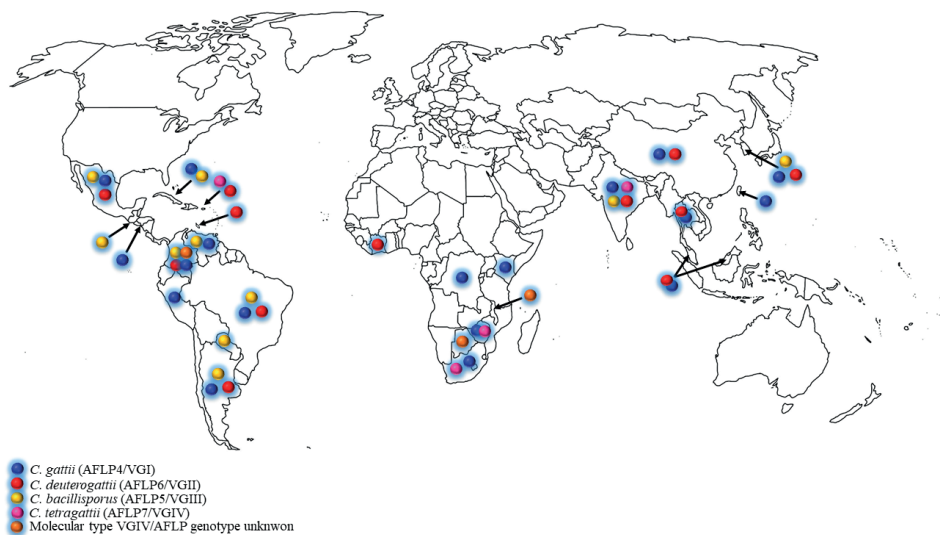


Fig 1. Distribution of clinical and environmental *Cryptococcus gattii* species complex in developing countries.

Table 1. *Cryptococcus gattii* species complex in developing countries.

| Continent | Species | Source | Country |
|-----------------------|-------------------------|------------------|----------------------------|
| Latin America | <i>C. gattii</i> s.s. | Clinical | Argentina [29,32-34] |
| | | Environmental | Brazil [21-23,31] |
| | | Veterinary | Colombia [24,35] |
| | | | Cuba [30] |
| | | | Honduras [27] |
| | | | Mexico [25,26] |
| | | Peru [28] | |
| | <i>C. deuterogattii</i> | Clinical | Brazil [18,21,22,35,36-38, |
| | | Environmental | 40,41,43-46] |
| | | Veterinary | Colombia [24,35] |
| | | | French Guiana [39] |
| | | | Mexico [25,26] |
| | | | Puerto Rico [42] |
| | <i>C. bacillisporus</i> | Clinical | Argentina [33] |
| | | Environmental | Brazil [21] |
| | | | Colombia [24,35,49] |
| | | Cuba [48] | |
| | | Guatemala [48] | |
| | | Mexico [25,26] | |
| | Paraguay [48] | | |
| | Venezuela [48] | | |
| <i>C. tetragattii</i> | Environmental | Colombia [28] | |
| | | Mexico [25,26] | |
| | | Puerto Rico [42] | |

Table 1. Continued

| | | | |
|---------------|-------------------------|---------------------------|---|
| Africa | <i>C. gattii s.l.</i> | Clinical Environmental | Botswana [53] Rwanda [54] South Africa [50-52,59] Zambia [55] |
| | <i>C. gattii s.s.</i> | Clinical | D.R. Congo [27] Kenya [56] South Africa [59] Zimbabwe [57,58] |
| | <i>C. deuterogattii</i> | Clinical | Ivory Coast [60] |
| | <i>C. tetragattii</i> | Clinical | Botswana [61] Malawi [61] South Africa [57] Zimbabwe [57,58] |
| Asia | <i>C. gattii s.l.</i> | Clinical Environmental | China [63,64,65] India [66-71] Taiwan [62] |
| | <i>C. gattii s.s.</i> | Clinical Environmental | China [78-82] India [74,83,84] Korea [77] Malaysia [72,73] Taiwan [75] Thailand [8,76] |
| | <i>C. deuterogattii</i> | Clinical | China [78,81,83] India [85] Korea [77,86] Malaysia [72,73] Thailand [76,87] |
| | <i>C. bacillisporus</i> | Clinical Environmental | India [84] Korea [77,86] |
| | <i>C. tetragattii</i> | Clinical Veterinary | India [88] |

s.s.: *sensu stricto*; *s.l.*: *sensu lato*.

Latin America

C. gattii sensu stricto (*s.s.*) (genotype AFLP4/VGI) is a major aetiologic agent of cryptococcosis among immunocompetent patients from Brazil [25–27], Colombia [29], Mexico [33, 34], Honduras [32], and Peru [35] and has caused pneumonia in a renal transplant patient from Argentina [21]. This pathogen has also been involved with a fatal infection in Cuba in an imported cheetah from South Africa [31]. In nature, this species was found in Psittaciformes excreta in Brazil [28] and some tree species, such as *Tipuana tipu*, *Grevillea robusta*, and *Eucalyptus* spp. in Argentina [22–24] and *Ficus* spp. in Colombia [30].

Cryptococcus deuterogattii (genotype AFLP6/VGII) has been isolated from clinical samples and has been involved in meningitis, cutaneous diseases, and lung infection in immunocompetent and HIV-positive patients from Brazil [25, 26, 36–38], Colombia [29], Mexico [33, 34], and French Guiana [45]. From Brazil, it was also reported causing disease in dogs [39, 40]. *Cryptococcus deuterogattii* is found in a variety of ecological niches, being isolated from tree detritus in Puerto Rico [46], from *Moquilea tomentosa*, *Plathymenia reticulata*, and *Senna sianea* in Brazil [18, 41, 42] and from *Eucalyptus* spp. in Colombia [30]. In addition, *C. deuterogattii* was also isolated from indoor dust from typical wooden houses in Amazonas, Brazil, and from *Guettarda acreana* trees [43, 44].

The species *C. bacillisporus* (genotype AFLP5/VGIII) has been isolated from clinical samples from Colombian immunocompetent patients [29], as well as from patients in Mexico [33, 34], Cuba [88], Brazil [25], Guatemala, Paraguay, and Venezuela [48]. In the environment, *Corymbia ficifolia* and *Ficus* spp. trees have been reported as reservoirs in Colombia [30, 47] and *Tipuana tipu* in Argentina [23].

Cryptococcus tetragattii (genotype AFLP7/VGIV) has been found in Puerto Rico from tree detritus [46] and the molecular type VGIV has also been found in México and Colombia, but AFLP or MLST genotyping has not been performed to differentiate *C. tetragattii* (genotype AFLP7/VGIV) and *C. decagattii* (genotype AFLP10/VGIV) from each other [33–35]. Some of these isolates have been recently investigated and shown to belong to *C. decagattii* rather than to *C. tetragattii* [1].

Africa

On the African continent, most of the literature data consists in descriptions of the *C. neoformans* species complex's ecological distribution and clinical involvement. But only few studies have found new *C. gattii* species complex members. In South Africa, HIV-positive and HIV-negative children and adults were reported to have cryptococcosis caused by *C. gattii s.l.*, but no genotyping was performed to determine the species [51–53]. The same holds true for Botswana [49] and Rwanda, where cryptococcal meningitis cases with *C. gattii s.l.* were found [50]. Environmental niches of *C. gattii s.l.* were investigated in Zambia, where positive samples were found in *Colophospermum mopane*, *Julbernardia globiflora*, *Eucalyptus* spp., *Brachystegia* spp., fig tree, and feces from Hyrax midden, but no genotyping was performed [54].

Cryptococcus gattii s.s. (genotype AFLP4/VGI) has been recovered from HIV-positive patients, bird droppings, *Acacia xanthophloea*, and *Eucalyptus saligna* from Kenya [55], from HIV-positive patients with meningitis from Zimbabwe [57, 58], D.R. Congo [32], and South Africa [56]. The species *C. deuterogattii* (genotype AFLP6/VGII) was reported causing cryptococcosis in HIV-positive patients from Ivory Coast [59]. In addition, *C. tetragattii* (genotype AFLP7/VGIV) was found to be a major cause of meningitis in HIV-positive patients in Zimbabwe [57, 58] and was reported to cause cryptococcosis in patients from Botswana, Malawi, and South Africa [57, 60]. A South African veterinary *C. tetragattii* (genotype AFLP7/VGIV) isolate was closely related to environmental *C. tetragattii* isolates from Colombia, Puerto Rico, and Spain [57].

Asia

There have been many studies performed in developing Asian countries reporting *C. gattii s.l.* causing human diseases and the ecological niche of this species complex. Unfortunately, most of the isolates were not genotyped. Chen and colleagues (2000) performed a study in Taiwan with clinical cases of cryptococcosis during the 1980s and 1990s [70]. Infections by *C. gattii s.l.* occurred in 35.6% of patients during the study period. The cryptococcosis cases included both immunocompetent and immunocompromised patients with a predominance of central nervous system (CNS) diseases [70]. In China, *C. gattii s.l.* was isolated from a surgical wound [61]. In addition, in India, it was isolated from HIV-positive and HIV-negative patients [62, 63]. Environmental niches of *C. gattii s.l.* in India were recognized being tree hollows of *Syzygium cumini*, *Ficus religiosa*, *Polyalthia longifolia*, *Azadirachta indica*, *Cassia fistula*, *Mimusops elengi*, and *Cassia marginata* [64–68], and flowers, bark, and detritus of *Eucalyptus camaldulensis* and *E. tereticornis* [69].

Cryptococcus gattii s.s. (genotype AFLP4/VGI) was isolated from patients with meningitis in Malaysia [80, 81] and India [76]; this species was also reported in clinical samples from Korean, Taiwanese, and Thai patients [79, 82, 83]. China has reported cryptococcosis cases in HIV-positive, HIV-negative, and immunocompetent patients [71–75]. The environmental source of Thai *C. gattii s.s.* is decaying wood inside a *Castanopsis argyrophylla* hollow [8], and in India this species was isolated from tree hollows [77, 78].

Cryptococcus deuterogattii (genotype AFLP6/VGII) has been isolated from chronic meningitis in Malaysia [80, 81], HIV-negative patients from Korea and India [79, 84, 85], and immunocompetent patients in China [71, 74, 75]. In Thailand, this species was reported causing disease in HIV-positive and HIV-negative patients, as well as causing primary cutaneous cryptococcosis [83, 86]. However, the environmental niche of this species has not been reported.

Cryptococcus bacillisporus (genotype AFLP5/VGIII) was found causing diseases in patients from Korea [79, 85], which is interesting because in Asia *C. gattii* s.s. and *C. deuterogattii* have a predominance among clinical samples. In India, the first environmental *C. bacillisporus* isolate was recovered from decaying wood of *Manilkara hexandra* [78].

Cryptococcus tetragattii (genotype AFLP7/VGIV) was isolated in India from several clinical sources, including an HIV-positive patient with meningitis, cutaneous lesions, and granulomas in HIV-negative patients. All these isolates were genetically similar to *C. tetragattii* found in Botswana, Africa. However, only one patient had previously travelled to Egypt [87].

A hypothesis to explain the differences in geographic distribution of *C. gattii*/*C. neoformans* species complexes was put forward by Casadevall and colleagues (2017). These authors hypothesized that it may be attributed to the breakup of the supercontinent Pangea. The physical separation of *Cryptococcus* species complexes was an important point for its speciation [89]. In addition, it was suggested that environmental events, such as wind, ocean currents, and animals, would be involved, driving the more recent speciation of *Cryptococcus* species complexes [89]. Despite all epidemiological studies carried out, there are many countries where the presence of cryptococcal molecular genotypes has not yet been explored [90].

Antifungal susceptibility among the *C. gattii* species complex

Among cryptococcal species, different antifungal susceptibility patterns have been observed. In general, the *C. gattii* species complex shows higher minimum inhibitory concentrations (MICs) of azoles than isolates from the *C. neoformans* species complex [68, 91, 92]. In addition, *C. gattii* s.l. clinical isolates from Taiwan showed higher amphotericin B and flucytosine MIC values than *C. neoformans* s.l. clinical isolates [70]. In Brazil, *C. deuterogattii* (genotype AFLP6/VGII) clinical isolates showed higher MIC values for flucytosine [93, 94] and fluconazole than *C. neoformans* s.s. (genotype AFLP1/VNI) [93].

However, different antifungal susceptibility profiles are also present within species of the *C. gattii* species complex. *Cryptococcus deuterogattii* (genotype AFLP6/VGII) has higher geometric mean MICs for flucytosine, fluconazole, voriconazole, itraconazole, posaconazole, and isavuconazole than *C. gattii* s.s. (genotype AFLP4/VGI) [95]. Lockhart and colleagues (2012) investigated the correlation of *C. gattii* species complex and its antifungal susceptibility [96].

Cryptococcus deuterogattii (genotype AFLP6/VGII) had the highest geometric mean MIC for fluconazole, followed by *C. bacillisporus* (genotype AFLP5/VGIII), genotype VGIV (AFLP non-genotyped), while *C. gattii s.s.* (genotype AFLP4/VGI) had the lowest among species [96]. Trilles and colleagues (2012) also observed that *C. deuterogattii* isolates had higher MICs of azoles than *C. gattii s.s.* [93]. An Indian study showed that clinical and environmental *C. gattii s.l.* isolates had high fluconazole MICs [68]. However, despite these differences in antifungal susceptibility among cryptococcal species, the initial cryptococcosis therapy is the same; the clinical management changes are according to presentations and immune status but does not consider the species involved in the disease [97].

Another important point is the phenomenon of heteroresistance, the ability of adaptation to high concentrations of drugs, observed in *C. gattii s.l.* to itraconazole and fluconazole [98, 99]. The development of heteroresistance is related to phenotypic changes, such as a decrease in cell and capsule size, low ergosterol content in the cell wall, less susceptibility to oxidative stress, and a great ability to proliferate inside macrophages [98, 99]. This intrinsic mechanism present in members of the *C. neoformans/C. gattii* species complexes may contribute to a relapse of cryptococcosis during maintenance therapy [98, 100]. However, the clinical importance of heteroresistance is not yet clear and requires further investigation [100, 101].

The *C. gattii* species complex: four molecular types, five genotypes or five species

The taxonomy of the tremellomycetous yeasts has recently been revised [102, 103]. Since the genus *Cryptococcus* was described, it has grown out as a highly polyphyletic one that contained more than 100 species within the orders Filobasidiales, Tremellales, and Trichosporonales [102, 103]. The taxonomic revision of the genus *Cryptococcus* has been extensively discussed over the past two decades. At the 6th International Conference on *Cryptococcus* and Cryptococcosis (ICCC) debate, "How many species and varietal states are there?" [104], different hypotheses were discussed about the status of the *C. neoformans/C. gattii* species complex: Should the situation be kept in a "two-species division"? [105, 106]. Should it be divided into six species? [107, 108]. Eight? [109]. The hypotheses were supported based on different opinions about the definition of species. The first one was supported by the idea that phenetic, biological, and cladistic species concepts need to be used together to properly classify the agents of cryptococcosis, because genetic variation as shown by the molecular types does not always reflect their biological characteristics [105, 106, 110]. However, the second hypothesis was based on phylogenetic support that included analysis of

mitochondrial, ribosomal, and nuclear genes to investigate the relationship among the various *C. neoformans* and *C. gattii* genotypes. The different genotypes clustered in six monophyletic lineages for all loci studied, suggesting that *C. neoformans* serotype A and D represent two different species and that *C. gattii* genotypes represent four individual taxa [107, 108]. The third hypothesis goes a little further, considering that each genotype within *C. neoformans* and *C. gattii* has sufficient genotypic variation to be considered a different species [109].

Phenotypic diversity within the *C. gattii* species complex is also supporting the division of five species. Capsule and cell size showed to be variable within the complex, *C. gattii* s.s. (genotype AFLP4/VGI) had the largest capsules but smaller cells compared to the other species, while *C. deutero-gattii* (genotype AFLP6/VGII) has the largest cells but smaller capsules [111]. All species in the *C. gattii* species complex have the ability to grow at 25, 30, and 35 °C, but with variable tolerance to 37 °C [1, 111]. *Cryptococcus deutero-gattii* (genotype AFLP6/VGII) has the highest thermotolerance to 37 °C, while *C. gattii* s.s. (genotype AFLP4/VGI), *C. bacillisporus* (genotype AFLP/VGIII), and *C. tetra-gattii* (genotype AFLP7/VGIV) have less growth at 37 °C than 30 °C [1, 111, 112]. There is no significant difference in tolerance to oxidative or osmotic stresses among species [111, 112].

The understanding of genetic diversity is an important step for the discovery of previously unrecognized phenetic differences [111]. The exact moment that individuals in an ancestral species are split into progeny species is not recognized for any method of species delimitation, because this process needs time until the changes in morphology, mating behavior, or gene sequences may be recognized in the progeny species [113]. Phylogenomic analyses calculated the time since divergence of the *C. neoformans* species complex and the *C. gattii* species complex to be ~34 million years ago (mya) [114]; the divergence between *C. deutero-gattii* (AFLP6/VGII) and the other species of the *C. gattii* species complex occurred ~12 mya [114, 115]; and the divergence of *C. neoformans* (AFLP1/VNI/AFLP1; VNII/AFLP1A/1B) and *C. deneoformans* (VNIV/AFLP2), ~24 mya [115]. The divergence among the species within the *C. neoformans*/*C. gattii* species complexes occurred recently and will most likely continue as an ongoing process. The occurrence of interspecies hybrids may also be attributed to the recent divergence event, because species currently hybridizing are most likely the youngest [116].

Although a revision of the cryptococcal taxonomy has been published, part of the cryptococcal research community is not fully in favor of using the 'seven species recognition'. Some investigators believe that it will lead to taxonomic instability due

to the fact that there are most likely more species present. Many points have been discussed, including the number of isolates used, the use of phylogenetic approaches for species delineation, the accommodation of hybrids in the new taxonomy, and the fact that the new names may cause confusion between the published literature and clinical practice [110]. With these points of view, Kwon-Chung and colleagues (2017) suggested the use of the “*C. neoformans* species complex” and the “*C. gattii* species complex” as an intermediate step, instead of using the seven species nomenclature, until biological and clinically relevant differences become clear [110]. Although, according to Hagen and colleagues (2017), it is important to consider the presence of different species inside the complexes to avoid delay in the clinical progress [117].

Final remarks

Clinical and environmental occurrence of the *C. gattii* species complex is related to geographic location, which may be attributed to the (micro)climate, or even a lack of diagnosis/environmental isolation. Cryptococcosis in most developing countries is underreported and the precise burden of cryptococcosis caused by the *C. gattii* species complex is uncertain. In addition, not all clinical laboratories differentiate the pathogenic *Cryptococcus* species. In the environment, many tree species have been described as a reservoir, proving that the *C. gattii* species complex has no tree species-specific relation, and is widely spread in the environment.

Cryptococcus gattii species complex members differ in phenotypic traits, as capsule and cell size, thermotolerance and antifungal susceptibility. Many studies have demonstrated higher MICs of azoles for members of the *C. gattii* species complex compared to the *C. neoformans* species complex. Difference in antifungal susceptibility has also been observed within the *C. gattii* species complex, with *C. deuterogattii* (genotype AFLP6/VGII) being less susceptible to azoles than *C. gattii s.s* (genotype AFLP4/VGI). However, in vitro antifungal susceptibility does not correlate to in vivo susceptibility. Clinical manifestations in patients with *C. gattii s.l.* infections tend to be more severe than *C. neoformans*. In the former, cerebral involvement causes more hydrocephalus, focal CNS signs, as well as papilledema, ataxia, hearing loss, altered mentation, and neurological sequelae. Usually, meningoencephalitis caused by *C. gattii s.l.* is followed by higher intracranial pressures, sometimes irresponsible to multiple lumbar puncture and/or CNF shunts. Simultaneous pulmonary involvement in >50% of patients is also observed, and mass lesions (cryptococcomas) are associated to a prolonged clinical course and respond slowly to therapy.

A new taxonomy of the polyphyletic genus *Cryptococcus* has been published, including the medical important species complexes *C. neoformans* and *C. gattii*. In addition, there are different opinions about the new classification. The presence of genetic differences within the *C. gattii* species complex needs to be considered in future studies to correlate genotypic and phenotypic traits of each species to diseases clinical presentation.

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Author Contributions

Patricia F. Herkert and Ferry Hagen designed the study and wrote the first draft; Rosangela L. Pinheiro and Marisol D. Muro analyzed the data; Jacques F. Meis and Flávio Queiroz-Telles revised the paper. All authors contributed to the writing and approved the final manuscript.

Conflicts of Interest

The other authors declare no conflict of interest.

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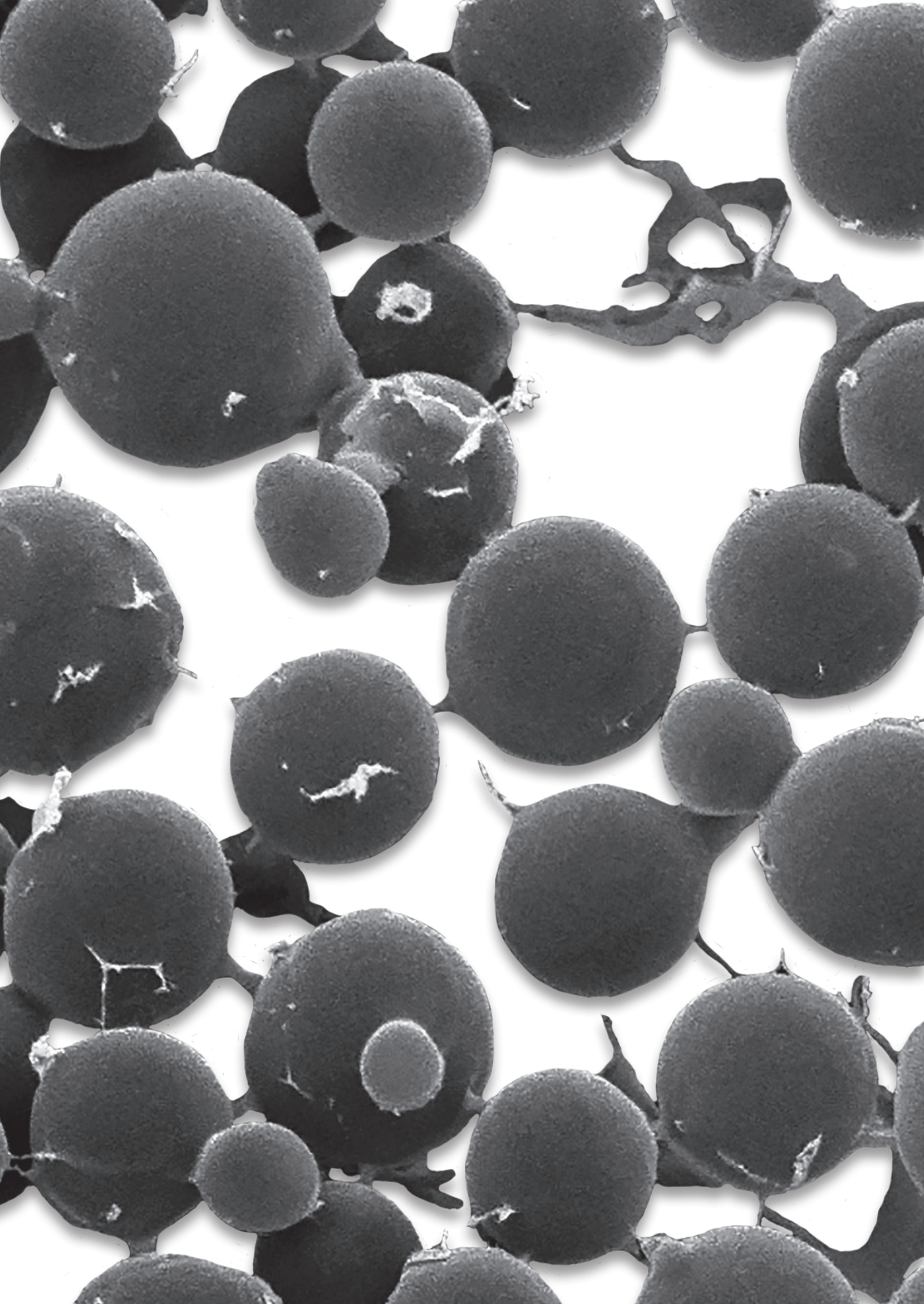
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Chapter 3

Molecular characterisation and antifungal susceptibility of clinical *Cryptococcus deuterogattii* (AFLP6/VGII) isolates from Southern Brazil

3

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Abstract

Cryptococcosis, caused by *Cryptococcus gattii sensu lato*, is an emerging disease that was initially found in (sub)tropical regions but recently expanded to temperate regions. *Cryptococcus gattii s.l.* infections are mostly encountered in healthy individuals, frequently affecting both lungs and the central nervous system (CNS). Usually, *C. gattii s.l.* is less susceptible to antifungal compounds than its counterpart, *C. neoformans s.l.* We studied 18 clinical *C. gattii s.l.* isolates with amplified fragment length polymorphism (AFLP) fingerprinting, mating-typing, multi-locus sequence typing (MLST) and antifungal susceptibility testing. All isolates were *C. deuterogattii* (genotype AFLP6/VGII), 14 were mating-type α and four were type **a**. Amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole showed high activity, with minimum inhibitory concentration (MIC) ranges of 0.063 – 0.25, 0.031 – 0.25, 0.031 – 0.25, 0.031 – 0.25 and $<0.016 - 0.25 \mu\text{g mL}^{-1}$, respectively. Fluconazole and flucytosine had high geometric mean MICs of 2.07 and $3.7 \mu\text{g mL}^{-1}$, respectively. Most cases occurred in immunocompetent patients ($n = 10$; 55.6 %) and CNS involvement was the most common clinical presentation ($n = 14$; 77.8 %). Three patients (16.7 %) showed sequelae, hyperreflexia, dysarthria, disidiadochokinesia, anosmia and upper limb weakness. In conclusion, all infections were caused by *C. deuterogattii* (AFLP6/VGII) and the majority of patients were immunocompetent, with the CNS as the most affected site. All antifungal drugs had high in vitro activity against *C. deuterogattii* isolates, except fluconazole and flucytosine.

Keywords: Cryptococcosis, *Cryptococcus gattii*, *Cryptococcus deuterogattii*, amplified fragment length polymorphism, multi-locus sequence typing, antifungal susceptibility.

Introduction

Cryptococcosis is caused by basidiomycetous yeast species that belong to the *Cryptococcus gattii*/*Cryptococcus neoformans* species complexes, mainly affecting lungs and the central nervous system (CNS) [1]. The taxonomy of the polyphyletic genus *Cryptococcus* has been thoroughly revised [2–4]. The two varieties of *C. neoformans* were recognised as species, with *C. neoformans* (=formerly *C. neoformans* variety *grubii*) and *C. deneoformans* (=formerly *C. neoformans* variety *neoformans*) [2]. The five *C. gattii* species complex genotypes were raised to the species level as *C. gattii sensu stricto* (AFLP4/VGI), *C. bacillisporus* (AFLP5/VGIII), *C. deuterogattii* (AFLP6/VGII), *C. tetragattii* (AFLP7/VGIV) and *C. decagattii* (AFLP10/VGIV) [2]. *Cryptococcus gattii s.s.* and *C. deuterogattii* are the main culprits of infections in immunocompetent hosts [2, 5], whereas *C. bacillisporus*, *C. tetragattii* and *C. decagattii* are commonly associated with immunocompromised hosts [2, 6–8].

Members of the *C. gattii* species complex have increasingly been reported from temperate climate regions [5, 9–11]. Trading of tree products, transport of propagules through water currents and via animals, insects and humans may be responsible for the world-wide spread [12, 13]. The first environmental isolation of *C. gattii s.l.* was reported from *Eucalyptus camaldulensis* [14]. Globally, *C. gattii s.l.* has been found on a plethora of other species of trees [13, 15–19].

In Europe, *C. gattii s.s.* is the most frequently encountered species, while few cases of *C. deuterogattii* infection are travel-related [20–22]. In Asia and Australia, *C. gattii s.s.* and *C. deuterogattii* are predominant [23]. *Cryptococcus bacillisporus* is rarely encountered outside North and South America [21, 23, 24], while *C. tetragattii* has mainly been isolated from Africa and India [8, 23]. On the American continent, *C. deuterogattii* is the predominant species among clinical and environmental isolates [23]. In North America, *C. deuterogattii* subgenotypes (AFLP6A/VGIIa, AFLP6B/VGIIb and AFLP6C/VGIIc) were reported as the cause of expanding outbreaks [5, 9, 10, 25]. A recent study showed that, by using coalescence gene genealogy analysis, the ancestral lineage of *C. deuterogattii* originated from South America, specifically from the Brazilian Amazon Rainforest, where mating-types α and α were reported in nearly equal rates, providing evidence of recombination within the Brazilian *C. deuterogattii* population [25].

Cryptococcus gattii s.l. is less susceptible to common antifungal compounds than *C. neoformans*; fluconazole and flucytosine show lower in vitro activity against *C. deuterogattii* strains [26–28]. In addition, heteroresistance to azoles in the

C. neoformans/C. gattii species complexes is an intrinsic mechanism that may contribute to relapse of cryptococcosis during maintenance therapy [29, 30].

In the present study, our aim was to compare the clinical outcome with molecular and antifungal susceptibility data of Brazilian *C. gattii s.l.* isolates.

Materials and methods

Isolates and clinical data

Eighteen clinical *C. gattii s.l.* isolates were isolated at the Hospital de Clínicas, Curitiba, PR, Brazil between 1999 and 2015. A single colony was taken for further microbiological and molecular characterisation. Medical records were accessed to collect clinical information.

Molecular characterisation

Extraction of genomic DNA, mating-type determination, amplified fragment length polymorphism (AFLP) fingerprinting and multi-locus sequence typing (MLST) were performed as described previously [20–22, 31].

Sequences were compared with those deposited in the MLST database (<http://mlst.mycologylab.org>). The alignment was performed with the online MAFFT alignment module [32] and visual inspection by MEGA version 7 [33], followed by a 1000× bootstrapped maximum likelihood analysis on an MLST dataset that comprises all known *C. deuterogattii* sequence types [8].

Antifungal susceptibility testing

Broth microdilution testing [34] included amphotericin B (Bristol Myers Squibb, Munich, Germany), fluconazole (Pfizer, Sandwich, United Kingdom), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), voriconazole (Pfizer), posaconazole (Merck, Kenilworth, NJ, USA), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland) and flucytosine (ICN Pharmaceuticals, Zoetermeer, The Netherlands). The concentration ranges were 0.016 – 16 µg mL⁻¹ for amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole, and 0.062 – 64 µg mL⁻¹ for fluconazole and flucytosine. *Cryptococcus* isolates were cultured onto Sabouraud dextrose agar for 48 h at 30 °C and the inocula were adjusted to 1 × 10³ CFU/mL in 0.9 % NaCl to perform the test. The microdilution plates were incubated at 35 °C for 72 h and the minimal inhibitory concentrations (MICs) were defined as the lowest concentration that produced complete growth inhibition for amphotericin B and

prominent decrease of growth (50 %) for other antifungal agents when compared with the drug-free growth control. *Candida parapsilosis* ATCC22019 and *C. krusei* ATCC6258 were used as quality controls [34]. The interpretation of MIC values was based on the epidemiological cut-off value (ECV) [26–28]. MIC₅₀ and MIC₉₀ values were obtained by ordering the data for each antifungal in ascending order and selecting the median and 90th quantile, respectively. Geometric mean MICs were calculated using Microsoft Office Excel 2010 software (Microsoft, Redmond, WA, USA). When the MIC was higher or less than the dilutions tested, 1 log₂ dilution higher or 1 log₂ dilution lower was used to calculate the geometric mean.

Results

Molecular characterisation

Mating-type analysis showed that 14 isolates were mating-type α and four were type **a**. MLST analysis showed that all 18 isolates clustered with the *C. deuterogattii* AFLP6/VGII reference strain WM178 (Fig. 1). Maximum likelihood analysis showed that most isolates were related with other Brazilian isolates, while isolate UFU986 was genetically indistinguishable from the outbreak genotype AFLP6A/VGIIa (Fig. 1). MLST sequences were deposited in GenBank with the accession numbers KU642658–KU642671, KU642673–KU642676 (CAP59), KU642703–KU642716, KU642718–KU642721 (GPD1), KU642748–KU642761, KU642763–KU642766 (IGS1), KU642793–KU642806, KU642808–KU642811 (LAC1), KU642838–KU642851, KU642853–KU642856 (PLB1), KU642883–KU642896, KU642898–KU642901 (SOD1) and KU642928–KU642938, KU642940–KU652944, KU642946, KU642966 (URA5). By AFLP genotyping analysis, four isolates clustered together with the *C. deuterogattii* AFLP6/VGII reference strain WM178, while the remaining isolates clustered in two distinct clades due to the presence of several dominant markers (Fig. 2).

Antifungal susceptibility profiles

The MIC values are presented in Table 1. Itraconazole, voriconazole, posaconazole, isavuconazole and amphotericin B showed high activity against *C. deuterogattii*. Fluconazole and flucytosine had the highest geometric mean MICs of 2.07 and 3.7 $\mu\text{g mL}^{-1}$, respectively.

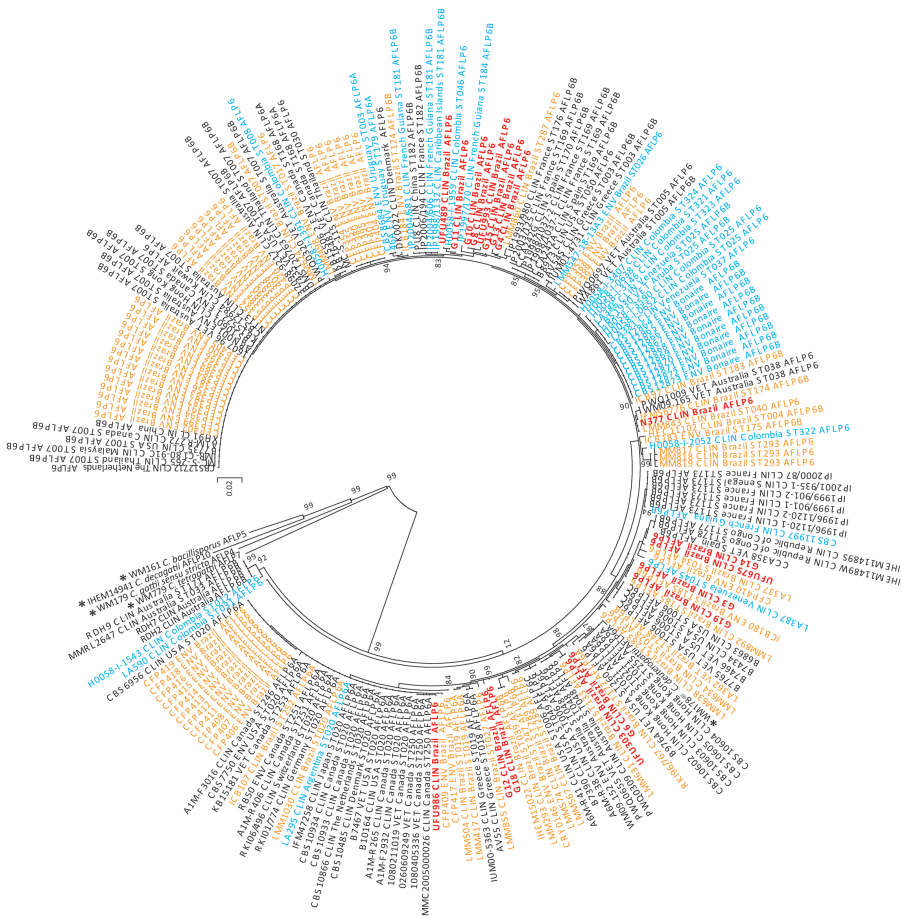


Fig. 1 Phylogenetic 1000x bootstrapped maximum likelihood analysis of *Cryptococcus deuterogattii* isolates based on the ISHAM consensus MLST dataset. *Cryptococcus gattii sensu stricto* (WM179), *C. bacillisporus* (WM161), *C. tetragattii* (WM779) and *C. decagattii* (HEM14941) were used as an outgroup. Bold red, isolates from the present study; orange, other Brazilian isolates; blue, other Latin American isolates; black, global isolates; *, reference strains



| Isolate# | Mating-type | Sex | Age (y) | Occupation | Immune status | Source | Treatment | Outcome | City (State) | Year |
|----------|-------------|-----|---------|----------------------|-----------------|------------|---------------------|------------------|---------------------------|------|
| UFU993 | a | M | 72 | Farmer | Immunocompetent | Blood/Skin | AMB (1) / FLC (400) | Died | Uberlândia (MG) | 2014 |
| WM178REF | - | - | - | - | - | - | - | - | - | - |
| UFU003 | alpha | F | 30 | Housewife | Immunocompetent | CNS | AMB (1) / FLC (400) | Cured | Uberlândia (MG) | 2015 |
| UFU986 | alpha | M | 55 | - | Immunocompetent | CNS | AMB (1) | Died | Uberlândia (MG) | 2008 |
| G6 | alpha | M | - | - | - | Lungs | - | - | - | 2005 |
| G18 | alpha | F | 20 | Supermarket employee | Immunocompetent | CNS/Lungs | ISA (200) | Cured / Sequelae | Almirante Tamandare (PR) | 2012 |
| G3 | alpha | M | 51 | Seller | Immunocompetent | CNS/Lungs | AMB (1) / ITC (100) | Died | Curitiba (PR) | 2002 |
| G12 | a | M | - | - | - | CNS | - | - | Curitiba (PR) | 2009 |
| G4 | alpha | M | 76 | - | - | Skin | - | - | Curitiba (PR) | 2005 |
| G10 | alpha | F | - | - | Immunocompetent | CNS | - | - | Curitiba (PR) | 2009 |
| N377 | alpha | F | 45 | Housekeeper | HIV-infected | CNS | AMB (1) / FLC (400) | Died | Curitiba (PR) | 2006 |
| G13 | alpha | M | 60 | Retired | Immunocompetent | CNS | AMB (1) / FLC (400) | Died | Colombo (PR) | 2010 |
| G14 | a | M | 32 | Lumberjack | Immunocompetent | CNS/Lungs | AMB (3) / FLC (600) | Cured / Sequelae | Almirante Tamandare (PR) | 2010 |
| G19 | alpha | F | 35 | Farmer | Immunocompetent | CNS/Lungs | ISA (200) | Cured | São José dos Pinhais (PR) | 2013 |
| UFU489 | alpha | F | 31 | Housewife | HIV-infected | CNS | AMB (1) / FLC (400) | Cured | Uberlândia (MG) | 1999 |
| G15 | alpha | M | 48 | Retired | HIV-infected | CNS | AMB (1) / FLC (600) | Cured | Curitiba (PR) | 2011 |
| UFU975 | a | M | 50 | - | HIV-infected | CNS | AMB (1) / FLC (400) | Died | Uberlândia (MG) | 2007 |
| G11 | alpha | M | 38 | Mason | Immunocompetent | CNS/Lungs | AMB (3) / FLC (400) | Cured / Sequelae | Pinhais (PR) | 2009 |
| G8 | alpha | - | - | - | - | Blood | - | - | Curitiba (PR) | 2008 |

Fig. 2 Amplified fragment length polymorphism (AFLP) fingerprint patterns of 18 Brazilian *C. deuterogattii* (AFLP6/VGI) isolates, including clinical and epidemiological characteristics of the patients. WM178 (*C. deuterogattii*/AFLP6/VGI) serves as a reference strain; AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; ISA, isavuconazole; treatment doses, AMB (mg/kg/day) and FLC, ITC and ISA (mg/day); -, data not available; F, female; M, male; CNS, central nervous system. PR, Paraná State; MG, Minas Gerais State

Table 1. Minimal inhibitory concentrations (MICs) of all the *Cryptococcus deuterogattii* isolates studied

| Isolate Code | Minimal Inhibitory Concentration ($\mu\text{g mL}^{-1}$) | | | | | | |
|-------------------|--|--------|------------|------------|------------|-------------|--------|
| | AMB | FLC | ITC | VOR | POS | ISA | 5FC |
| G3 | 0.063 | 0.5 | 0.063 | 0.063 | 0.063 | 0.031 | 4 |
| G4 | 0.125 | 8 | 0.25 | 0.25 | 0.25 | 0.25 | 8 |
| G6 | 0.031 | 8 | 0.125 | 0.125 | 0.125 | 0.063 | 2 |
| G8 | 0.031 | 2 | 0.125 | 0.063 | 0.063 | 0.063 | 4 |
| G10 | 0.125 | 4 | 0.125 | 0.125 | 0.125 | 0.125 | 8 |
| G11 | 0.063 | 0.5 | 0.031 | 0.031 | 0.031 | <0.016 | 1 |
| G12 | 0.063 | 0.5 | 0.063 | 0.063 | 0.063 | 0.063 | 4 |
| G13 | 0.031 | 4 | 0.063 | 0.063 | 0.063 | 0.063 | 2 |
| G14 | 0.063 | 4 | 0.125 | 0.125 | 0.125 | 0.125 | 4 |
| G15 | 0.125 | 4 | 0.125 | 0.125 | 0.125 | 0.125 | 4 |
| G18 | 0.125 | 2 | 0.125 | 0.063 | 0.063 | 0.063 | 4 |
| G19 | 0.125 | 16 | 0.25 | 0.25 | 0.25 | 0.25 | 4 |
| N377 | 0.125 | 0.5 | 0.063 | 0.063 | 0.063 | 0.031 | 8 |
| UFU303 | 0.125 | 8 | 0.125 | 0.125 | 0.125 | 0.063 | 4 |
| UFU489 | 0.063 | 0.5 | 0.063 | 0.063 | 0.063 | 0.031 | 2 |
| UFU675 | 0.125 | 0.5 | 0.063 | 0.063 | 0.063 | 0.063 | 4 |
| UFU986 | 0.063 | 4 | 0.25 | 0.125 | 0.125 | 0.125 | 4 |
| UFU993 | 0.125 | 1 | 0.063 | 0.063 | 0.063 | 0.063 | 4 |
| MIC range | 0.063-0.25 | 0.5-16 | 0.031-0.25 | 0.031-0.25 | 0.031-0.25 | <0.016-0.25 | 1-8 |
| MIC ₅₀ | 0.063 | 2 | 0.125 | 0.063 | 0.063 | 0.063 | 4 |
| MIC ₉₀ | 0.125 | 8 | 0.25 | 0.125 | 0.125 | 0.125 | 8 |
| Geometric mean | 0.0788 | 2.0785 | 0.0994 | 0.0853 | 0.0853 | 0.0651 | 3.7034 |

AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; VOR, voriconazole; POS, posaconazole; ISA, isavuconazole; 5FC, flucytosine.

Clinical data

The majority of isolates were recovered from immunocompetent patients ($n = 10$; 55.6 %). Fourteen (77.8 %) had CNS involvement and four (22.2 %) of them were HIV-positive. Furthermore, six patients (33.3%) had lung infection, including five (27.7 %) with associated CNS symptoms, while two patients (11.1 %) had cutaneous cryptococcosis. Three (16.7 %) developed neurological sequelae after the cryptococcal infection, one left-sided hyperreflexia, dysarthria and disdiadochokinesia, one had motor sequelae in the upper limbs and one developed

anosmia. Nine patients (50 %) were treated with a combination of amphotericin B and fluconazole, one received amphotericin B and itraconazole, one amphotericin B and two were treated with isavuconazole. Two patients received liposomal amphotericin B after development of nephrotoxicity. Treatment dosing and outcome data are summarised in Fig. 2.

Discussion

Infections caused by members of the *C. gattii* species complex have a wide spectrum of clinical presentations, including pneumonia, meningoencephalitis, skin lesions and pulmonary and cerebral cryptococcoma formation, and surgical intervention may be necessary in certain cases [6, 35–38]. CNS disease is associated with neurological sequelae such as visual impairment, deafness, limb weakness and dysphasia [39]. We observed hyperreflexia, dysarthria, disdiadochokinesia, anosmia and upper limb weakness. Some studies reported that 60–90% of patients with *C. gattii* species complex cryptococcosis have pulmonary symptoms with or without CNS involvement [10, 39, 40]. These data differ from this study, where the majority of patients ($n = 14$; 77.8 %) had CNS disease, five (27.7 %) also had lung involvement and a solitary patient (5.5 %) had only a lung infection.

All isolates were found to be *C. deuterogattii*, which is in concordance with the literature data, as it is predominant among clinical, veterinary and environmental isolates in Brazil [18, 41–50]. *Cryptococcus gattii* s.s. is more often reported in southern Brazil [51, 52] and the southeastern region [52, 53], and *C. bacillisporus* in the southeastern and northeastern part [52]. Despite increasing epidemiological surveys in Brazil, information about the species distribution are fragmented and underrepresented from several Brazilian states. This is mainly due to the lack of proper diagnosis and the absence of an efficient reporting system [43].

We found that four out of 18 *C. deuterogattii* were mating-type **a**. The presence of both mating types within the South American *C. deuterogattii* population demonstrates that recombination events are a common phenomenon, causing high genetic diversity compared to other localities [25]. Globally, the majority of clinical isolates were found to be mating-type α , and with respect to *C. deuterogattii*, this is also the case for the ongoing outbreaks in North America [6, 25]. Recently, the first Australian *C. deuterogattii* mating-type **a** isolate was reported [54]. The absence or low number of mating-type **a** isolates within study populations suggests that *C. deuterogattii* is either clonal or reproduces by same-sex mating [55].

Antifungal susceptibility testing showed that amphotericin B had high activity, which is in concordance with other studies [27, 48, 56–58]. Fluconazole had the lowest activity among the triazoles tested, with an MIC range of 0.5 – 16 $\mu\text{g mL}^{-1}$. Nevertheless, all MIC values were in the susceptible range [26, 59]. Hagen et al. [58] observed lower fluconazole activity against all *C. gattii* species complex members, with European and North American isolates showing higher MIC values (0.125 to $>64 \mu\text{g mL}^{-1}$ and 0.5 – 64 $\mu\text{g mL}^{-1}$, respectively). A recent Brazilian study reported high MICs (2 – 64 $\mu\text{g mL}^{-1}$), with a geometric mean of 6.08 $\mu\text{g mL}^{-1}$ for *C. deuterogattii* [60]. We also observed low MICs of itraconazole and new triazoles [26, 27, 56, 58].

Isavuconazole is a second-generation triazole antifungal, with a broad spectrum of activity against many important fungal pathogens [61]. Antifungal susceptibility tests have shown potent in vitro activity against members of *C. neoformans/C. gattii* species complexes, even for those that are less susceptible to fluconazole [28, 62, 63]. We observed that isavuconazole had excellent activity against *C. deuterogattii*, showing the lowest geometric mean MIC (0.065 $\mu\text{g mL}^{-1}$) among the antifungal compounds tested. Two immunocompetent female patients were included in a multi-centre international clinical trial with isavuconazole (ClinicalTrials.gov, NCT00634049) [64]. The first was 20 years old and working in a supermarket, where she had continuous contact with wood, fruits and vegetables. After the diagnosis, she received amphotericin B deoxycholate therapy for 16 days, but without response. The therapy was switched to isavuconazole, resulting in a complete clinical, radiological and microbiological response after 6 months of treatment. Anosmia was observed as a sequel. The second patient was a pharmacist, who received isavuconazole as primary therapy for a total of 176 days, resulting in a complete response without any sequelae.

Flucytosine had the lowest in vitro activity, showing the highest geometric mean MIC (3.7 $\mu\text{g mL}^{-1}$) among the antifungals tested, but the MICs for all isolates were within the wild-type range. It is known that resistance to flucytosine is rare [58, 65], but the use as monotherapy could lead to acquired resistance [66]. There are studies showing less susceptibility to flucytosine in *Cryptococcus* [58, 67, 68], but since flucytosine is not registered in Brazil, we supposed that the patients did not receive previous treatment with this drug. Higher MICs of flucytosine were also reported in other Brazilian studies [45, 48, 53, 60].

In conclusion, the majority of cases occurred in immunocompetent patients, with the CNS as the most affected site. All infections were caused by *C. deuterogattii* (AFLP6/VGII) and all antifungal drugs had in vitro activity against this species, except fluconazole and flucytosine.

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Conflict of interest

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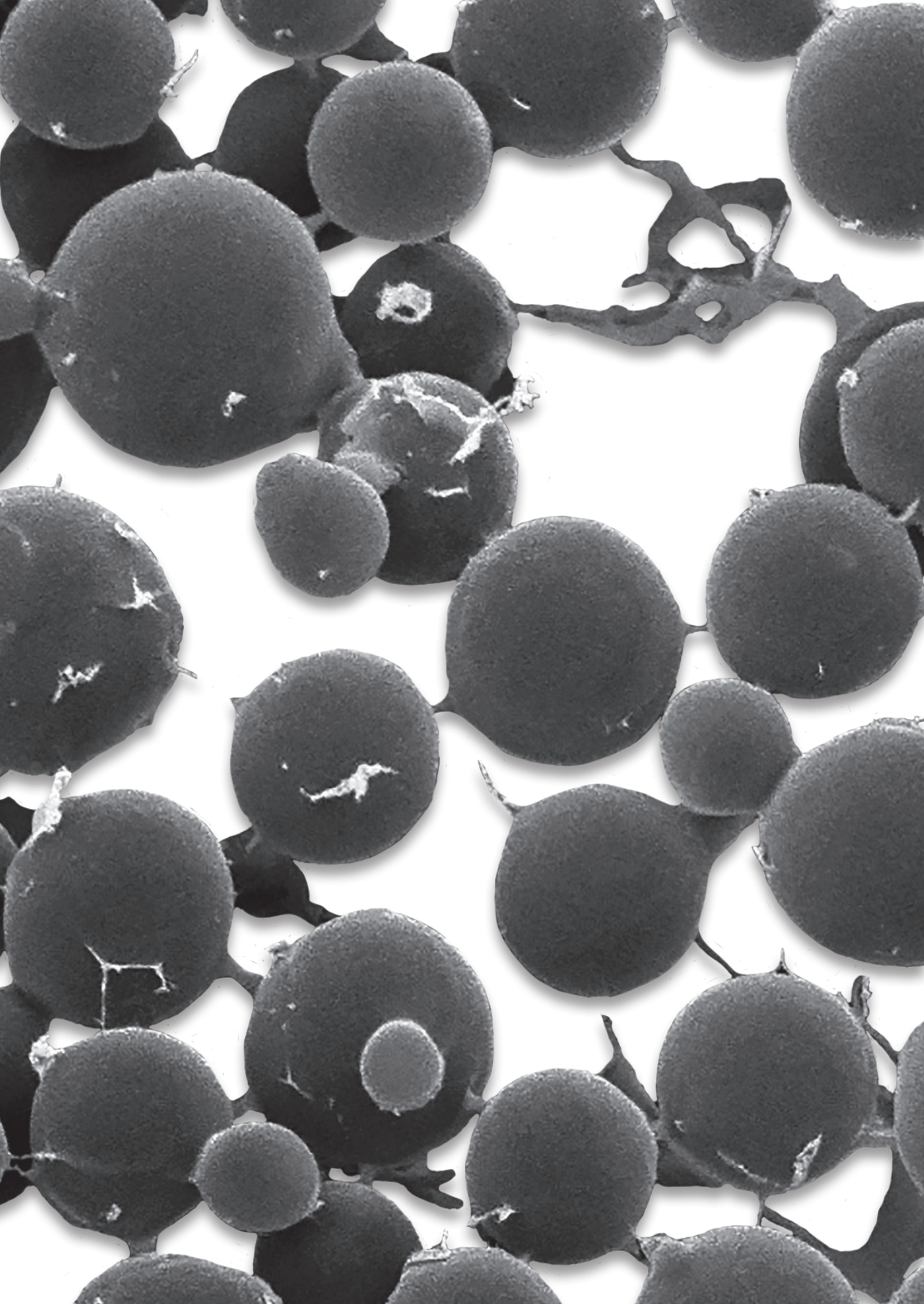
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Chapter 4

Differential *in vitro* cytokine induction by the species of *Cryptococcus gattii* complex

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Abstract

Cryptococcal species vary in capsule and cell size, thermotolerance, geographic distribution, and affected populations. *Cryptococcus gattii sensu stricto* and *C. deuterogattii* affect mainly immunocompetent hosts; however, *C. bacillisporus*, *C. decagattii*, and *C. tetragattii* cause infections mainly in immunocompromised hosts. This study aimed to compare the capacities of different species of the *C. gattii* species complex to induce cytokines and antimicrobial molecules in human peripheral blood mononuclear cells (PBMCs). *Cryptococcus bacillisporus* and *C. deuterogattii* induced the lowest levels of tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and IL-6 among the five species of the *C. gattii* complex. *Cryptococcus deuterogattii* induced higher levels of IL-22 than those induced by *C. tetragattii* and the environmental species *C. flavescens*. In addition, *C. bacillisporus* and *C. gattii sensu stricto* proliferated inside human monocyte-derived macrophages after 24 h of infection. All *Cryptococcus* species were able to generate reactive oxygen species (ROS) in human PBMCs, with *C. bacillisporus* and *C. deuterogattii* being more efficient than the other species. In conclusion, *C. bacillisporus* and *C. deuterogattii* induce lower levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 and higher ROS levels than those induced by the other species. Species of the *Cryptococcus gattii* complex have different abilities to induce cytokine and ROS production by human PBMCs.

Keywords *Cryptococcus*, *Cryptococcus gattii*, cryptococcosis, cytokines, proinflammatory

Introduction

Cryptococcosis is a systemic fungal disease caused by the basidiomycetous yeasts belonging to the *Cryptococcus neoformans*/*C. gattii* species complexes [1]. The infection is acquired from the environment by inhalation of desiccated yeast cells or basidiospores, which establish either an asymptomatic latent infection or pneumonia and meningoencephalitis [2]. The taxonomy of the polyphyletic genus *Cryptococcus* was recently revised [1, 3, 4]. The two *C. neoformans* varieties were raised to the species level, as *C. neoformans sensu stricto* (formerly *C. neoformans* var. *grubii*) and *C. deneoformans* (formerly *C. neoformans* var. *neoformans*) [1]. In addition, the five genotypes within the *C. gattii* species complex were raised to the species level, as *C. gattii sensu stricto* (AFLP4/VGI), *C. bacillisporus* (AFLP5/VGIII), *C. deuterogattii* (AFLP6/VGII), *C. tetragattii* (AFLP7/VGIV), and *C. decagattii* (AFLP10/VGIV) [1]. Although the taxonomic revision was established after >15 years of debate, part of the community prefers the use of the term “species complex” [5] despite the accumulating evidence for the revised taxonomy [6].

Species discrimination has clinical and epidemiological importance, as it has been observed that there are differences in disease presentation and in susceptibility profiles for antifungal drugs [1]. Infections by members of the *C. gattii* species complex mainly cause pneumonia, meningoencephalitis, skin lesions, pulmonary or cerebral cryptococcomas, and central nervous system (CNS) disease associated with neurological sequelae. These infections typically require prolonged antifungal therapy [7-11]. In contrast, severe meningitis is the main infection caused by *C. neoformans sensu lato* [12]. In addition, the species *C. gattii sensu stricto* and *C. deuterogattii* affect mainly immunocompetent hosts [1, 13], whereas *C. bacillisporus*, *C. decagattii*, and *C. tetragattii* are more commonly found in immunocompromised hosts, mostly HIV/AIDS patients, similar to *C. neoformans sensu lato* [1, 7, 14, 15]. However, fatal cryptococcosis by *C. bacillisporus* in immunocompetent hosts has been reported in the literature [16, 17].

In addition, phenotypic diversity supports the differences among the species within the *C. gattii* species complex. Capsule and cell sizes vary: *C. gattii sensu stricto* (AFLP4/VGI) has the largest capsules but smaller cells than those of other species, whereas *C. deuterogattii* (AFLP6/VGII) has the largest cells but smaller capsules [18]. All species have the ability to grow at 25, 30, and 35°C, but they have various levels of tolerance to 37°C [1, 18]. *Cryptococcus deuterogattii* (AFLP6/VGII) has the highest thermotolerance at 37°C, while *C. gattii sensu stricto* (AFLP4/VGI), *C. bacillisporus*

(AFLP/VGIII), and *C. tetragattii* (AFLP7/VGIV) have less growth at 37°C than at 30°C [1, 18, 19]. There are no significant differences in tolerance to oxidative [19] or osmotic [18] stresses among species.

An important aspect of cryptococcal infections is how *Cryptococcus* species evade the host immune system and establish infection. To reach the CNS, cryptococcal cells use paracytosis to move between tight junctions of the brain endothelium [20], transcytosis to move directly through the endothelial cells [21], and hitchhiking within phagocytes [22, 23]. Typically, these fungi avoid killing by host phagocytic cells due to virulence factors, such as the polysaccharide capsule, melanin, and urease production, in addition to other traits, such as the ability to escape phagocytosis by inducing nonlytic exocytosis and by producing titan cells [24-26].

Cryptococcus gattii sensu lato is also able to disturb the inflammatory process, inducing low levels of proinflammatory cytokine tumor necrosis factor alpha (TNF- α) production *in vitro* [27]. The cryptococcal capsule components glucuronoxylomannan and galactoxylomannan dampen inflammation by suppressing the NF- κ B pathway and locking surface antigen recognition [27, 28]. In consequence, dendritic cell maturation is compromised, leading to release of TNF- α , interleukin-12 (IL-12), and IL-23 at low levels and to decreased major histocompatibility complex class II molecule expression. Thus, suboptimal T-cell responses and weak proinflammatory responses are induced, inhibiting adequate cryptococcal clearance [27-29].

Most knowledge about the immune response patterns against *Cryptococcus* species is derived from studies of *C. neoformans* species complex isolates performed with animal models and human cells [30-34]. However, the interest in studying immune response patterns against the members of the *C. gattii* species complex has increased recently [28, 29, 35-38] due to evidence of genetic diversity, phenetic differences, and epidemiologic particularities among the species [1, 18, 39-41]. Based on these aspects, the present study hypothesized that the five species within the *C. gattii* complex have different abilities to induce cytokine production in human cells, a crucial step for the activation of host defense. In addition, macrophage infection and antimicrobial molecules were evaluated.

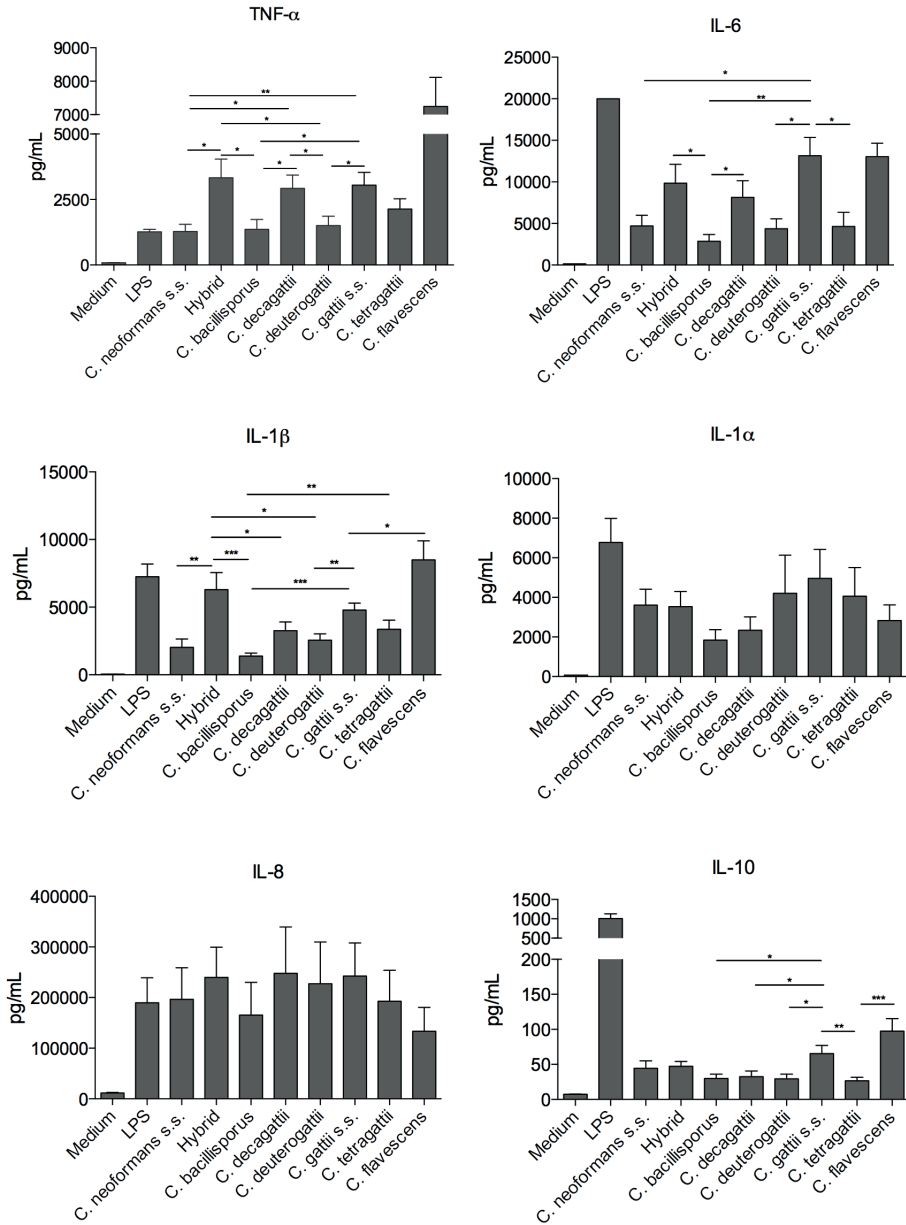


Fig 1. Monocyte-derived cytokine production induced by members of the *C. gattii* species complex. Human PBMCs ($n = 6$) from healthy volunteers (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml) or live yeast forms of *Cryptococcus* species (2.5×10^6 cells/ml). After 24 h of incubation, TNF- α , IL-1 α , IL-1 β , IL-6, IL-8, and IL-10 production in supernatants was determined by ELISA. Mean values with SEM for three independent experiments done in duplicate are presented. *, $P \geq 0.05$; **, $P \geq 0.01$; ***, $P \geq 0.001$. s.s., *sensu stricto*.

Results

***Cryptococcus bacillisporus* and *C. deuterogattii* induced lower levels of TNF- α , IL-1 β , and IL-6 than those induced by the other species**

The quantitative concentrations of cytokines produced by human peripheral blood mononuclear cells (PBMCs) from healthy volunteers after stimulation with different *Cryptococcus* species are shown in Fig. 1. *Cryptococcus neoformans sensu stricto*, *C. bacillisporus*, and *C. deuterogattii* induced lower levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 than those induced by the other species. *Cryptococcus gattii sensu stricto* and the control species *C. flavescens* induced higher levels of IL-10 than those observed with the other species, and no differences in IL-8 levels were observed.

Cryptococcus deuterogattii* induced higher levels of IL-22 than those induced by *C. tetragattii* and *C. flavescens

A comparison of quantitative T-cell-derived cytokine production stimulated by *Cryptococcus* species is shown in Fig. 2. *Cryptococcus* species induced low levels of gamma interferon (IFN- γ) production by human PBMCs, without significant differences among the species. The same was observed for IL-17, with all species tested inducing small amounts of IL-17. For IL-22, *C. bacillisporus*, *C. decagattii*, *C. deuterogattii*, and *C. gattii sensu stricto* induced similar levels. However, *C. deuterogattii* induced higher levels than those induced by *C. tetragattii* ($P \geq 0.01$) and *C. flavescens* ($P \geq 0.01$).

***Cryptococcus bacillisporus* and *C. gattii sensu stricto* proliferated inside macrophages after 24 h of infection**

Human primary granulocyte-macrophage colony stimulating factor (GM-CSF)-derived macrophages were used to evaluate the rates of phagocytosis of three *Cryptococcus* species. *Cryptococcus neoformans sensu stricto* was included as a control; *C. bacillisporus* and *C. gattii sensu stricto* were included because the first induced low and the latter large amounts of proinflammatory cytokines. The rates of phagocytosis for macrophages infected with *C. neoformans sensu stricto*, *C. bacillisporus*, and *C. gattii sensu stricto* are shown in Fig. 3. After infection, 60 to 80% of the total number of macrophages were infected with cryptococci. No significant differences in the percentage of infection were observed after 3 or 24 h of incubation. After preactivation of macrophages with IFN- γ (50 U/ml), no effect on phagocytosis rates was observed (Fig. 3A), and no differences in numbers of intracellular cryptococci per preactivated or nonpreactivated macrophage were found. However, after 24 h, there was a significant increase in the number of intracellular *C. neoformans*, *C. bacillisporus*, or

C. gattii sensu stricto organisms per macrophage ($P \geq 0.05$), independent of the absence or presence of IFN- γ . The infection index after 24 h was lower for *C. gattii sensu stricto* ($P \geq 0.05$) than for *C. neoformans* and *C. bacillisporus* in preactivated and nonpreactivated macrophages. Photomicrographs of macrophages infected with different cryptococcal species are presented in Fig. 3B to D.

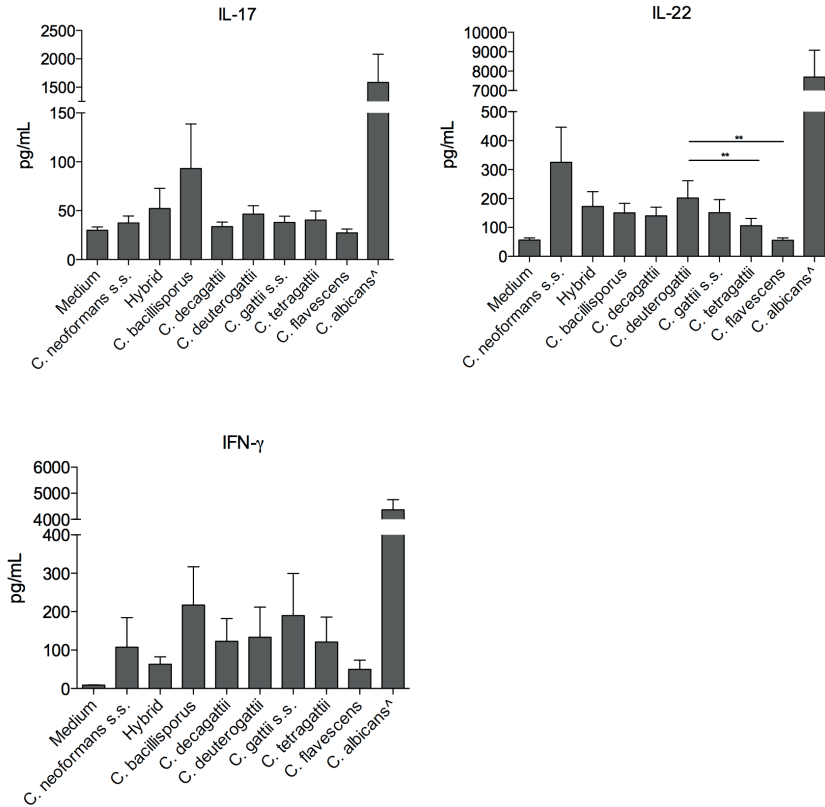


Fig 2. T-cell-derived cytokine production induced by members of the *C. gattii* species complex. Human PBMCs ($n = 6$) from healthy volunteers (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml), live yeast forms of *Cryptococcus* species (2.5×10^5 cells/ml), or heat-killed *Candida albicans* (1×10^7 cells/ml), as a positive control. After 7 days of incubation, IL-17, IL-22, and IFN- γ production in supernatants was determined by ELISA. Mean values ($n = 6$) with SEM for three independent experiments done in duplicate are presented. *, $P \geq 0.05$; **, $P \geq 0.01$. \wedge , *Candida albicans*.

The phagocytosed cryptococci were recovered by macrophage lysis, and their viability was investigated by CFU counting (Fig. 3E). The numbers of viable *Cryptococcus neoformans sensu stricto* and *C. bacillisporus* organisms were higher

after 24 h than after 3 h of infection for nonpreactivated macrophages. For preactivated macrophages, there was an increase in the number of viable cells only for *C. bacillisporus* after 24 h of infection compared to 3 h of infection. In contrast, *C. gattii sensu stricto* had more viability than that of *C. bacillisporus* 3 h after infecting nonpreactivated macrophages. Nevertheless, the viability of *C. gattii sensu stricto* decreased from 3 h to 24 h. Comparing the viabilities among species, *C. neoformans* had more viability than *C. bacillisporus* and *C. gattii sensu stricto* after 24 h of infection in preactivated or nonpreactivated macrophages ($P \geq 0.05$).

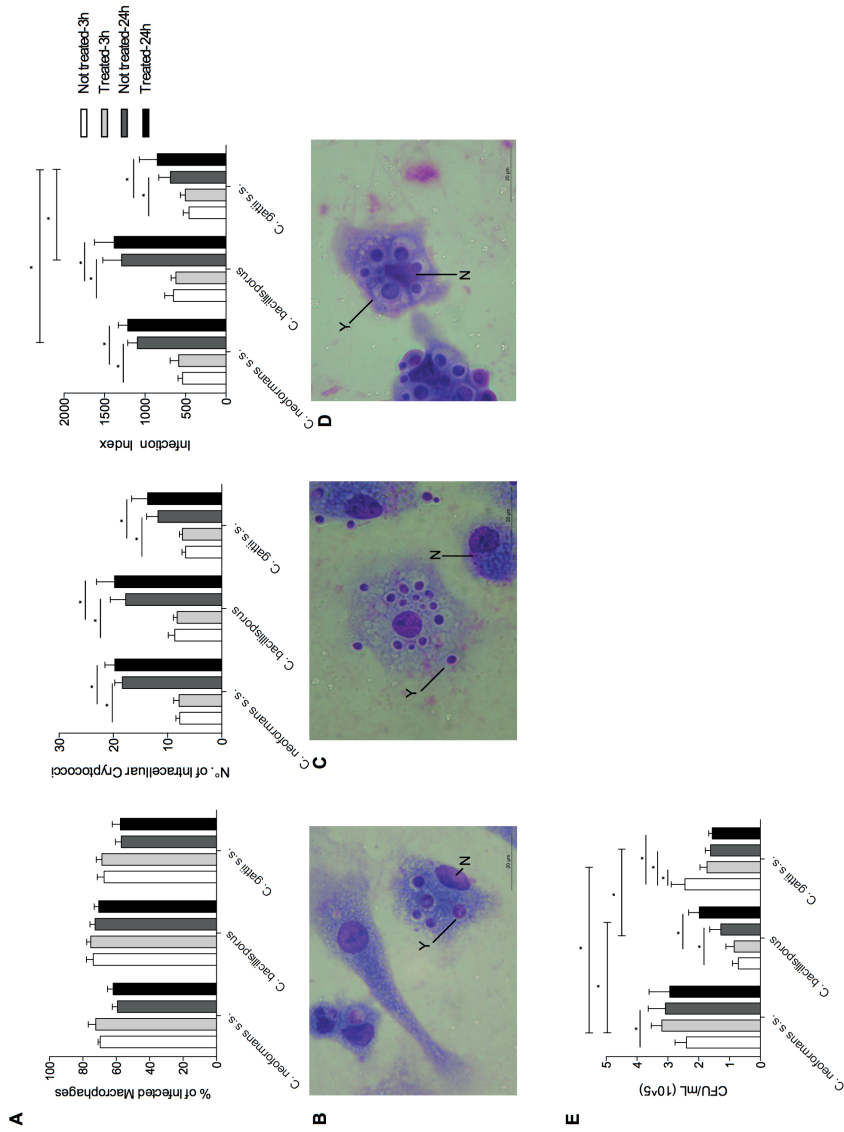
Macrophage cytokine production was measured in the supernatants 3 and 24 h after infection. After 3 h of infection, there was no production of IL-1 β , IL-6, and IL-10 by macrophages. All tested cryptococcal species induced IL-8 and TNF- α production in macrophages after 24 h; *C. gattii sensu stricto* induced higher levels of IL-8 than those induced by *C. bacillisporus* ($P \geq 0.05$) (see Fig. S1 in the supplemental material). Preactivated macrophages with IFN- γ produced higher levels of TNF- α after 24 h of infection with *C. neoformans sensu stricto* than those produced by nonpreactivated macrophages infected with *C. neoformans sensu stricto* ($P \geq 0.05$); however, no differences were found among other species.

***Cryptococcus bacillisporus* and *C. deuterogattii* induced higher levels of ROS production than those induced by the other species**

All species within the *C. gattii* complex induced smaller amounts of reactive oxygen species (ROS) than those seen with *Candida albicans*. *Cryptococcus decagattii*, *C. gattii sensu stricto*, *C. tetragattii*, and an interspecies hybrid (*C. neoformans sensu stricto* \times *C. gattii sensu stricto*) showed the lowest levels of ROS. On the other hand, human PBMCs produced high levels of ROS when stimulated with *C. bacillisporus*, *C. deuterogattii*, and *C. flavescens*, with peak of ROS production at 11 to 19 min (Fig. 4B).

The mRNA expression levels of inducible nitric oxide synthase (iNOS) and the antimicrobial peptides β -defensin 2 and cathelicidin were measured quantitatively (Fig. S2). *Cryptococcus neoformans sensu stricto* downregulated and the *C. gattii* species complex did not change iNOS expression (Fig. S2A). Similarly, no change in β -defensin 2 mRNA expression was detected (Fig. S2B). Overall, the interspecies hybrid, *C. bacillisporus*, *C. decagattii*, and *C. gattii sensu stricto* downregulated the expression of cathelicidin in human PMBCs *in vitro* (Fig. S2C). The main results found are summarized in Fig. 5 and Table 1.

Fig 3. Macrophage infection indexes and cryptococcal viability. Primary human GM-CSF-derived macrophages ($n = 6$) (2×10^5 cells/ml), pretreated or not with IFN- γ (50 U/ml), were infected with one of three cryptococcal species (1×10^6 cells/ml). After 3 and 24 h, the lysates of cells diluted 100x in PBS were seeded onto Sabouraud dextrose medium and incubated for 48 to 72 h, and the numbers of CFU were determined. (A) Primary GM-CSF-derived macrophages (2×10^5 cells/ml) were infected with cryptococcal species (1×10^6 cells/ml) on coverslips under the conditions described above. After the incubation time, cells were stained, and the percentage of infected macrophages, number of intracellular cryptococci per macrophage, and infection index were determined. (B to D) *C. neoformans sensu stricto* (B), *C. bacillisporus* (C), and *C. gattii sensu stricto* (D) internalized by macrophages. (E) Total numbers of live cryptococcal cells recovered from macrophages after 3 and 24 h of incubation. **N**, macrophage nucleus; **Y**, yeast cell. *, $P \geq 0.05$.



Discussion

In this study, we demonstrate that species of the *C. gattii* complex present different abilities to induce cytokines in human cells. *Cryptococcus bacillisporus* and *C. deuterogattii* induced lower levels of production of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 by human PBMCs than those induced by the other species. *Cryptococcus gattii sensu stricto* induced higher levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 and higher levels of the anti-inflammatory cytokine IL-10 than those observed with other species of the *C. gattii* complex. The interspecies hybrid, *C. decagattii*, and *C. gattii sensu stricto* induced high levels of the proinflammatory cytokines TNF- α and IL-6. All cryptococcal species induced low levels of IFN- γ and IL-17 compared to those induced by another medically important yeast, *Candida albicans*, used as a positive control.

The decrease of proinflammatory cytokines may favor an environment that disturbs maturation and epitope presentation by dendritic cells, leading to suboptimal levels of the Th1 and Th17 responses, which are protective against cryptococcosis and provide fungal clearance [29, 30, 42, 43]. IL-1 α and IL-1 β are important in the host defense against fungal infections, activating innate immune cells and modulating adaptive immunity [44, 45]. In addition, high levels of the proinflammatory cytokines IL-6, IL-8, IFN- γ , and TNF- α in serum have been associated with cryptococcal meningitis survival in HIV patients [46]. Schoffelen and colleagues observed that heat-killed *C. gattii* species complex members induced higher concentrations of the proinflammatory cytokines IL-1 β , IL-6, TNF- α and IL-17/22 than those seen with the *C. neoformans* species complex [47]. However, they did not investigate differences among species of the *C. gattii* complex. Only *C. deuterogattii* induced significant production of IL-22, with clear differences among the species. IL-22 is a proinflammatory cytokine that promotes antimicrobial molecule production by epithelial cells and may have a protective role against infections [48], including cryptococcal infections. Meningitis caused by members of the *C. gattii* species complex in non-HIV-infected patients has been associated with low levels of the proinflammatory cytokines IFN- γ , TNF- α , and IL-6 and high levels of the anti-inflammatory cytokine IL-10 [49]. In the present study, *C. gattii sensu stricto* and the predominantly environmental species *C. flavescens* induced high levels of IL-10, while other species of the *C. gattii* complex induced low levels of IL-10. According to Angkasekwinai and coworkers, *C. gattii sensu stricto* and *C. deuterogattii* downregulate pulmonary chemokine expression, leading to a failure to mount protective immunity in immunocompetent hosts [29] and thus contributing to the disease process. It may be that differences in immune responses or clinical

aspects of immunocompetent hosts preferentially infected by *C. gattii sensu stricto* or *C. deuterogattii* are due to the differential levels of cytokines induced in PBMCs, as we showed that these species lead to production of different amounts of cytokines.

Our results showed that all cryptococcal species induced low levels of IFN- γ , and the use of IFN- γ in macrophages preactivation did not improve the phagocytosis rate or reduce the infection, even for *C. neoformans sensu stricto*. However, this cytokine increased TNF- α production in *C. neoformans sensu stricto*-infected human macrophages (see Fig. S1 in the supplemental material). Ikeda-Dantsuji and colleagues also showed that preactivation with IFN- γ did not promote phagocytosis of *C. deuterogattii* but that IFN- γ increased the phagocytic rate of *C. neoformans sensu stricto* [50]. Since IFN- γ has been associated with a good prognosis for cryptococcosis [46, 51, 52], these findings may be important for understanding the pathogenesis of cryptococcosis. In our experiments, IFN- γ did not significantly increase the TNF- α production of macrophages after infection with *C. bacillisporus* or *C. gattii sensu stricto*, which would affect the efficiency of macrophages at controlling cryptococcal growth. Wang and colleagues observed low levels of serum IFN- γ in immunocompetent patients with pulmonary cryptococcosis, and after antifungal treatment the serum levels of IFN- γ increased and the protective inflammatory response was restored. They suggested that cryptococcal infection may suppress the immune system and that its elimination helps to establish the immune system again [52]. In addition, the use of IFN- γ in combination with standard therapy has been related to successful fungal clearance, resolution of symptoms, and restoration of immunological parameters [51, 53]. Improvements in general condition were also observed among non-HIV/nontransplant patients after administration of IFN- γ as adjuvant therapy [54]. Meningitis caused by *C. gattii sensu lato* in non-HIV-infected patients has been associated with low levels of IFN- γ and other proinflammatory cytokines in parallel with high levels of the anti-inflammatory cytokine IL-10 [49]. Thus, according to *in vivo* results, IFN- γ is important for controlling cryptococcal disease. The results obtained in *in vitro* experiments in the present study may have been affected by the assay conditions, such as the IFN- γ concentration and the time of macrophage exposure to this cytokine. Despite that possibility, for PBMCs, no differences in IFN- γ induction could be detected among the species of the *C. gattii* species complex.

Cryptococcus bacillisporus induced the highest production of ROS, induced small amounts of cytokines, and downregulated cathelicidin compared to those observed with the other species. In contrast, *C. gattii sensu stricto* induced low levels of ROS and high levels of cytokines and downregulated cathelicidin expression. We speculate

that ROS are important for controlling *C. bacillisporus* infection during the first hours of infection, as fewer viable *C. bacillisporus* cells than *C. gattii sensu stricto* cells were recovered after 3 h of infection. However, after the initial killing by ROS, the nonkilled *C. bacillisporus* cells were able to proliferate inside macrophages, which might explain the increased number of intracellular cryptococci after 24 h of infection (Fig. 3E). It might be that the production of cytokines by the different *Cryptococcus* species does not correlate with the virulence of the particular species. However, the combination of cytokine production and ROS production may be important in the host-microbe interaction that finally results in control of the microorganism (in this case *C. bacillisporus*). Although *C. gattii sensu stricto* had more viable cells after 3 h of infection, we observed a decrease in viable intracellular cryptococci after 24 h. We speculate that nonlytic exocytosis may have happened, as cryptococcal cells were observed outside macrophages after 24 h of incubation [27]. For this species, the combination of ROS and proinflammatory cytokines may increase the capacity of macrophages to control infection. The environmental species *C. flavescens* induced high concentrations of ROS and cytokine production by human PBMCs, suggesting that the host immune response is effective at eliminating this nonhuman pathogen; this may explain the small number of cases of cryptococcosis caused by this species [55]. However, *C. neoformans* and *C. deuterogattii* induced small amounts of cytokines and large amounts of ROS in the first hour of infection, showing the ability to escape and survive inside macrophages, in accordance with previous studies [56, 57-61]. Hole and coworkers observed that ROS produced by human plasmacytoid dendritic cells are required for *C. neoformans sensu stricto* growth inhibition but are not the only mechanism used to control *C. neoformans sensu stricto* [62]. The control of the interspecies hybrid, *C. gattii sensu stricto*, and *C. decagattii* may be mediated by cytokine pathways in an ROS-independent manner. These species induced high concentrations of proinflammatory cytokines (TNF- α , IL-6, and IL-1 β) which are essential for protection against cryptococcosis [43, 44, 46]. According to our results, neither iNOS nor β -defensin 2 seems to be involved in *C. gattii* species complex infection control, but other antimicrobial molecules might be involved.

In conclusion, *C. bacillisporus* and *C. deuterogattii* induced low levels of production of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6, and *C. deuterogattii* induced more IL-22 than that induced by *C. tetragattii*. In contrast, *C. gattii sensu stricto* stimulated high levels of production of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 and high levels of the anti-inflammatory cytokine IL-10. Despite ROS being important for controlling *C. bacillisporus* infection at the beginning of infection, nonkilled *C. bacillisporus* cells are able to proliferate inside macrophages, maintaining the infection. On the other hand, for *C. gattii sensu*

stricto, the combination of ROS and proinflammatory cytokine production may be involved in infection control. Overall, the species within the *C. gattii* complex have different abilities to induce cytokine production and ROS by human PBMCs. We have to be aware that host variability is an important factor in determining disease processes, and we cannot exclude the possibility that differences in exposure time or concentrations of fungi or the variation in the host defense systems of the human donors may have affected the outcomes of the comparison experiments. Our results with regard to differences in cytokine and ROS production in response to the members of the *C. gattii* species complex might contribute to the understanding of the cryptococcosis disease process.

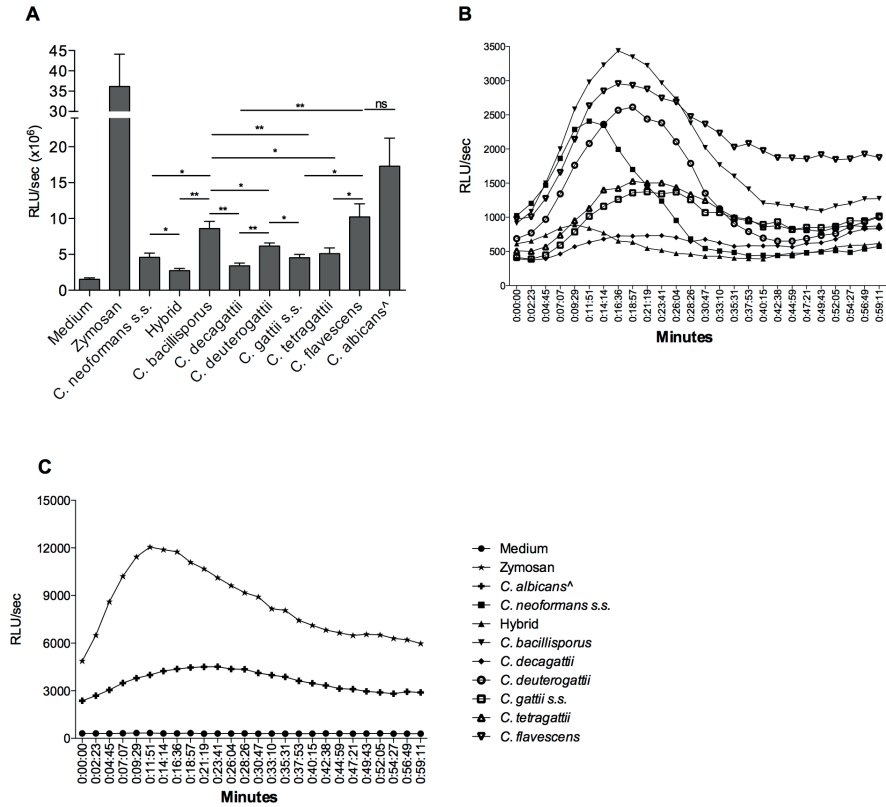


Fig 4. ROS production induced by cryptococcal species. Human PBMCs (1×10^6 cells/ml) from healthy volunteers were stimulated with zymosan (positive control; 1 mg/ml), live yeast forms of *Cryptococcus* species (5×10^6 cells/ml), or heat-killed *Candida albicans* (as a control; 1×10^7 cells/ml), and ROS production was measured by chemiluminescence assay. Data represent mean RLU ($n = 6$ [in quadruplicate]) for two independent experiments, with data collected every 2 min 23 s for 1 h. (A) Integral levels of PBMC ROS production induced by controls and *Cryptococcus* species. *, $P \geq 0.05$; **, $P \geq 0.01$; ns, not significant. (B) Kinetics of PBMC ROS production induced by *Cryptococcus* species. (C) Kinetics of PBMC ROS production induced by controls. [^], *Candida albicans*.

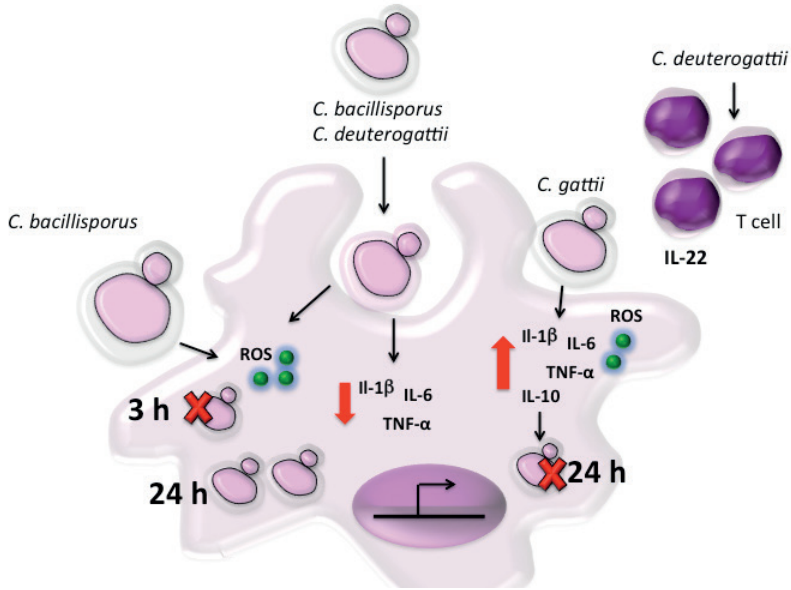


Fig 5. Representative scheme of cytokine profile and antimicrobial molecule production in human PBMCs induced by cryptococcal species. *C. bacillisporus* and *C. deuterogattii* induced low levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6, and *C. deuterogattii* also induced IL-22. In contrast, *C. gattii sensu stricto* induced higher levels of TNF- α , IL-1 β , IL-6, and IL-10 than those induced by the other species. ROS are important for controlling *C. bacillisporus* infection in the beginning, but nonkilled *C. bacillisporus* cells are able to proliferate inside macrophages. *Cryptococcus gattii sensu stricto* infection control may be mediated by the combination of ROS and proinflammatory cytokine production.

Table 1. Summary of observed data

| Species, Host | Regulation ^a | | | | | |
|---|-------------------------|-------|-------|-----|--------------|------------------------------|
| | Cytokine | IL-22 | IL-10 | ROS | Cathelicidin | Proliferation in macrophages |
| <i>C. gattii s.s.</i> , immunocompetent | ↑ | | ↑ | ↓ | ↓ | ↓ |
| <i>C. deuterogattii</i> , immunocompetent | ↓ | ↑ | | ↑ | | |
| <i>C. bacillisporus</i> , immunocompromised | ↓ | | | ↑ | | ↑ |
| <i>C. decagattii</i> , immunocompromised | ↑ | | | ↓ | | |
| <i>C. tetragattii</i> , immunocompromised | ↑ | | | ↓ | | |
| <i>C. neoformans</i> , immunocompromised | | | | ↓ | | |
| <i>C. flavescens</i> , environmental | | | ↑ | ↑ | | |

^a ↓, downregulation; ↑, upregulation.

Materials and Methods

Cryptococcus isolates

Eight cryptococcal strains were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands). The details of these isolates are provided in Table 2. The yeast isolates were grown on Sabouraud dextrose agar plates, and a suspension of each isolate was prepared in sterile phosphate-buffered saline (PBS), pH 7.4 (1,000 × g, 10 min, 10°C). The cell viability was checked by dilution in 0.01% trypan blue in PBS, and cells were counted by use of a hemocytometer. *Cryptococcus neoformans sensu stricto* was used as a control instead of *C. deneoformans* because it is predominant worldwide and is related to the majority of cryptococcosis cases caused by *C. neoformans* species complex infection [39, 64].

Candida isolate

Heat-killed *Candida albicans* ATCC MYA-3573 (UC 820) diluted in sterile PBS was used as a positive control for cytokine stimulation.

PBMC isolation and stimulation

Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Human peripheral blood mononuclear cell (PBMC) isolation was performed by dilution of blood in PBS and differential density centrifugation over Ficoll-Paque density gradient medium (GE Healthcare, Uppsala, Sweden). Cells were washed three times in sterile PBS and then suspended and cultured in RPMI 1640 medium (Gibco-Life Technologies, Carlsbad, CA, USA) supplemented with 10 mM pyruvate, 10 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA). Subsequently, cells were counted in a Coulter ZH counter (Beckman Coulter, Fullerton, CA, USA) and adjusted to 5×10^6 cells/ml. Thereafter, 100 µl aliquots of PBMCs (5×10^5 cells) were added to a round-bottomed 96-well plate and incubated with either 100 µl live *Cryptococcus* (2.5×10^6 cells/ml) isolate, 100 µl of ultrapure *Escherichia coli* lipopolysaccharide (LPS) (O111:B4; Sigma-Aldrich) (10 ng/ml), or 100 µl of RPMI medium alone at 37°C and 5% CO₂. All stimulations were performed in medium containing 10% human serum, which was obtained from a serum pool from healthy volunteers. After 24 h or 7 days, supernatants were collected and stored at –20 °C until they were used for assays. After 24 h, the cell monolayers were collected by adding 100 µl of 0.5% Triton X-100 to measure intracellular cytokine production and 200 µl of TRIzol and were stored at –80°C until mRNA extraction.

Cytokine measurements

Monocyte-derived TNF- α , IL-6, IL-1 β , IL-8, and IL-10 levels in culture supernatants after 24 h of incubation were determined by use of commercial enzyme-linked immunosorbent assay (ELISA) kits (Sanquin, Amsterdam, the Netherlands [IL-6, IL-8, and IL-10], and R&D Systems, Minneapolis, MN, USA [TNF- α and IL-1 β]), and intracellular IL-1 β protein in cell lysates collected by use of Triton X-100 was measured by another ELISA (R&D Systems) according to the manufacturer's instructions. In preliminary experiments, we determined that 24 h was the time of maximum levels of monocyte-derived cytokine production for each species (data not shown). The levels of T-cell-derived cytokines IFN- γ , IL-17, and IL-22 in culture supernatants after 7 days of incubation were determined using ELISA kits (Sanquin [IFN- γ] and R&D Systems [IL-17 and IL-22]). Results are presented in picograms per milliliter.

Macrophage infection assay

Primary monocytes were obtained by hyperosmotic Percoll (Sigma-Aldrich) density gradient centrifugation of PBMCs. Afterward, cells were washed once with PBS and seeded in tissue culture plates at 37°C and 5% CO₂ in the presence of RPMI medium supplemented with 10% human pooled serum and GM-CSF (50 ng/ml) (R&D Systems). After 6 days of differentiation, macrophages were harvested by use of cold PBS and adjusted to 5×10^6 cells/ml. Thereafter, 200 μ l aliquots of macrophage suspension (2×10^5 cells/ml) were added to 24-well plates, with or without glass coverslips. Macrophages were left to adhere for 30 min and preincubated with or without recombinant human IFN- γ (rhIFN- γ) (50 U/ml; Boehringer Ingelheim, Alkmaar, the Netherlands) for 1 h. Thereafter, cultures were infected with 1×10^6 (multiplicity of infection [MOI] = 5:1) live yeast forms of *C. neoformans sensu stricto*, *C. bacillisporus*, or *C. gattii sensu stricto*. After 3 h, noninternalized fungi were washed off, the medium was replaced, and cultures were incubated for 24 h. For wells without coverslips, after the incubation time (3 h or 24 h), macrophages were lysed with water and mechanical lysis, diluted 100 \times in PBS, and seeded onto Sabouraud dextrose medium. Plates were incubated for 48 to 72 h at 37°C for CFU quantification. For wells with coverslips, cells were fixed, stained with Giemsa stain (Merck Millipore, Billerica, MA, USA), and analyzed by light microscopy (magnification, $\times 1,000$) to determine the infection index. Three hundred cells were analyzed, and the percentage of infected cells and the mean number of intracellular cryptococci per infected cell were determined. The infection index was calculated as follows: infection index = percentage of infected macrophages \times mean number of intracellular cryptococci per macrophage.

ROS measurement

Human PBMCs (1×10^6 cells/ml) were suspended in Hanks' balanced salt solution and exposed to different concentrations of live yeast forms of *Cryptococcus* species (5×10^6 cells/ml). Heat-killed *C. albicans* and zymosan (1 mg/ml) (InvivoGen, Toulouse, France) were used as positive controls. ROS formation was measured by a chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (5 mM; Sigma-Aldrich). The luminometer measured chemiluminescence in the integration mode at 37°C for 1 h after luminol was added. Results are presented as numbers of relative light units (RLU) per second.

mRNA expression by qPCR

RNA isolation was carried out as reported previously [65]. RNA was precipitated with isopropanol and washed with 75% ethanol, followed by reconstitution in RNase-free water. Subsequently, RNA was reverse transcribed into cDNA by use of an iScript kit (Bio-Rad, Hercules, CA, USA). Diluted cDNA was used for quantitative real-time PCR (qPCR) analysis, which was done by using a StepOne Plus sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR green master mix (Applied Biosystems). Primer sequences (see Table S1 in the supplemental material) for inducible nitric oxide synthase (iNOS), β -defensin 2, and cathelicidin were obtained from the Harvard Primerbank database. Primers were purchased from Biolegio (Nijmegen, the Netherlands). The mRNA analysis was done by the $2^{-\Delta\Delta CT}$ method, and the results were normalized against those for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene.

Table 2. Details of cryptococcal strains used to conduct human PBMC and macrophage stimulation

| Isolate Code | Other collections | Species | Mating-Serotype | Genotype | Origin | Reference |
|--------------|------------------------|---|-----------------|-----------------------|---------------------------|--|
| CBS 10515 | H99; WM 04.15 | <i>C. neoformans</i> s.s. | αA | AFLP1/VNI | Clinical – U.S.A. | Hagen et al. 2015 [1]; CBS [^] |
| CBS 10085 | WM148 | <i>C. neoformans</i> s.s. | | AFLP1/VNI | Clinical – Australia | |
| CBS996 | - | <i>C. neoformans</i> s.s. | | AFLP1/VNI | Clinical – Argentina | |
| CBS 10496 | LSPQ#308 | <i>C. neoformans</i> s.s. × <i>C. gattii</i> s.s. | A × B | AFLP1/VNI × AFLP4/VGI | Clinical – Canada | Hagen et al. 2010 [63]; CBS [^] |
| CBS 10081 | WM161; TP 0689; D1.13H | <i>C. bacillisporus</i> | αB | AFLP5/VGIII | Clinical – U.S.A. | Hagen et al. 2015 [1]; CBS [^] |
| CBS6993 | - | <i>C. bacillisporus</i> | | AFLP5/VGIII | Clinical – U.S.A. | |
| CBS8735 | - | <i>C. bacillisporus</i> | | AFLP5/VGIII | Environmental, Colombia | |
| CBS 11687 | IHEM14941; IHEM14941S | <i>C. decagattii</i> | - | AFLP10/VGIV | Clinical – Mexico | Hagen et al. 2015 [1]; CBS [^] |
| IHEM14941W | - | <i>C. decagattii</i> | | AFLP10 | Clinical – Mexico | |
| CBS 10082 | WM 178; IFM 50894 | <i>C. deuterogattii</i> | αB | AFLP6/VGII | Clinical – Australia | Hagen et al. 2015 [1]; CBS [^] |
| CBS6936 | - | <i>C. deuterogattii</i> | | AFLP6/VGII | Clinical – U.S.A. | |
| CBS10090 | - | <i>C. deuterogattii</i> | | AFLP6/VGII | Clinical – Greece | |
| CBS 10078 | WM 179; H33.1; MH56 | <i>C. gattii</i> s.s. | αB | AFLP4/VGI | Clinical – Australia | Hagen et al. 2015 [1]; CBS [^] |
| CBS919 | - | <i>C. gattii</i> s.s. | | AFLP4/VGI | Clinical – U.S.A. | |
| CBS6290 | - | <i>C. gattii</i> s.s. | | AFLP4/VGI | Clinical – D.R. Congo | |
| CBS 10101 | WM 779; IFM 50896 | <i>C. tetragattii</i> | αC | AFLP7/VGIV | Veterinary – South Africa | Hagen et al. 2015 [1]; CBS [^] |
| B5472 | - | <i>C. tetragattii</i> | | AFLP7/VGIV | Clinical – India | |
| B5478 | - | <i>C. tetragattii</i> | | AFLP7/VGIV | Clinical – India | |
| CBS 8645 | DBVPG 7166 | <i>C. flavescens</i> | - | - | Clinical – Greece | CBS [^] |
| UC820 | ATCC MWA-3573 | <i>C. albicans</i> [†] | - | - | Clinical – U.S.A. | ATCC [‡] |

Statistical analysis

Data are given as mean values with standard errors of the means (SEM). The Mann-Whitney U test for unpaired, nonparametric data was used to compare differences in cytokine production between two groups. Two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test was used when more than two groups were compared. GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) was used to tabulate and analyze the data.

Ethics statement

The donation of blood by healthy volunteers was performed after written consent and was approved by the Ethical Committee of the Arnhem-Nijmegen Region, the Netherlands.

Supplemental material

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00958-17>. Supplemental file 1, PDF file, 0.3 MB.

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Supplemental material

Table S1. Primer sequences used to perform iNOS and antimicrobial molecules qPCRs.

| Primer | Sequence |
|-----------------------------|-----------------------------|
| GAPDH forward | 5'-AGGGGAGATTCAGTGTGGTG-3' |
| GAPDH reverse | 5'-CGACCACCTTTGTCAAGCTCA-3' |
| iNOS forward | 5'-GCGCAGACATGATCGCCATA-3' |
| iNOS reverse | 5'-CCTCACCGAACTCACCAGC-3' |
| Cathelicidin forward | 5'-TGCCAGGTCTCAGTAC-3' |
| Cathelicidin reverse | 5'-GTGACTGCTGTGTCCT-3' |
| β -defensin 2 forward | 5'-GGTGTTTTTGGTGTATAGGCG-3' |
| β -defensin 2 reverse | 5'-AGGGCAAAGACTGGATGACA-3' |

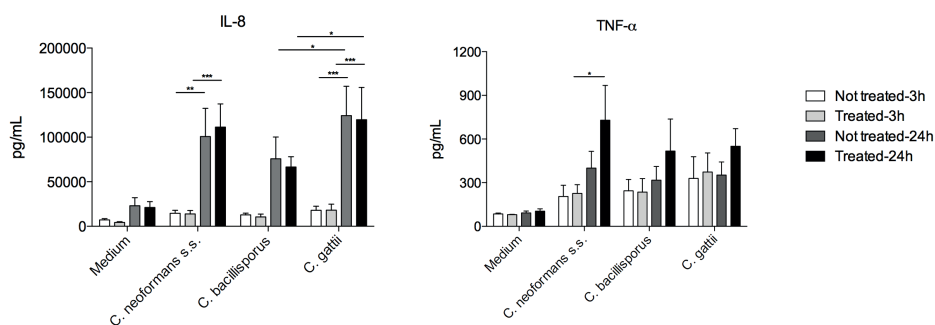


Fig S1. Macrophages cytokine production induced by *Cryptococcus* species. Human primary GM-CSF-derived macrophages ($n=6$) (2×10^5 cells/mL) were infected with live *Cryptococcus* species (1×10^6 cells/mL). After 3 and 24 h of incubation, TNF- α and IL-8 production was determined in supernatants by ELISA. Mean values with SEM from three independent experiments are presented. Two-way ANOVA Bonferroni posttests were used to calculate the differences among the treatments for each species. Statistical significance values considered were $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

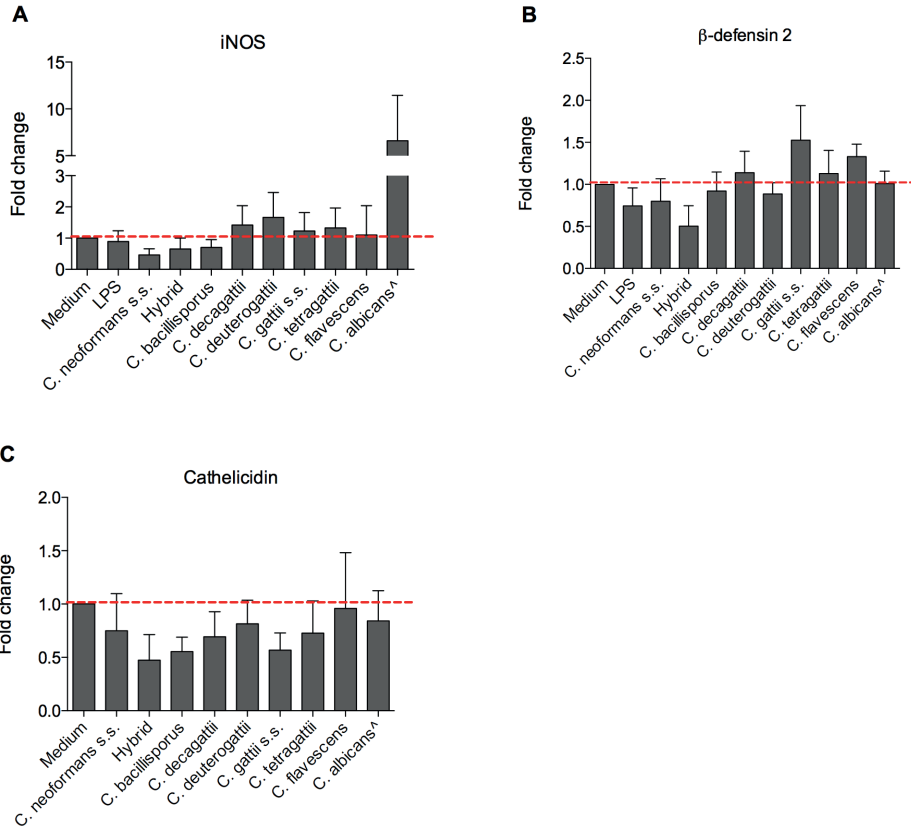


Fig S2. iNOS and antimicrobial molecules mRNA expression induced by *Cryptococcus* species. Human PBMCs ($n=6$) (5×10^5 cells/mL) from healthy volunteers were stimulated with yeas live forms of *Cryptococcus* species (2.5×10^5 cells/mL) and heat-killed *C. albicans* (1×10^7 cells/mL). After 24 h of incubation the mRNA was isolated and quantitative real-time PCR (qPCR) was performed to determine iNOS, β -defensin 2 and cathelicidin mRNA expression. Quantitative qPCR values are presented as fold change compared with non-stimulated cells (RPMI). Mean values with SEM from three experiments are presented.

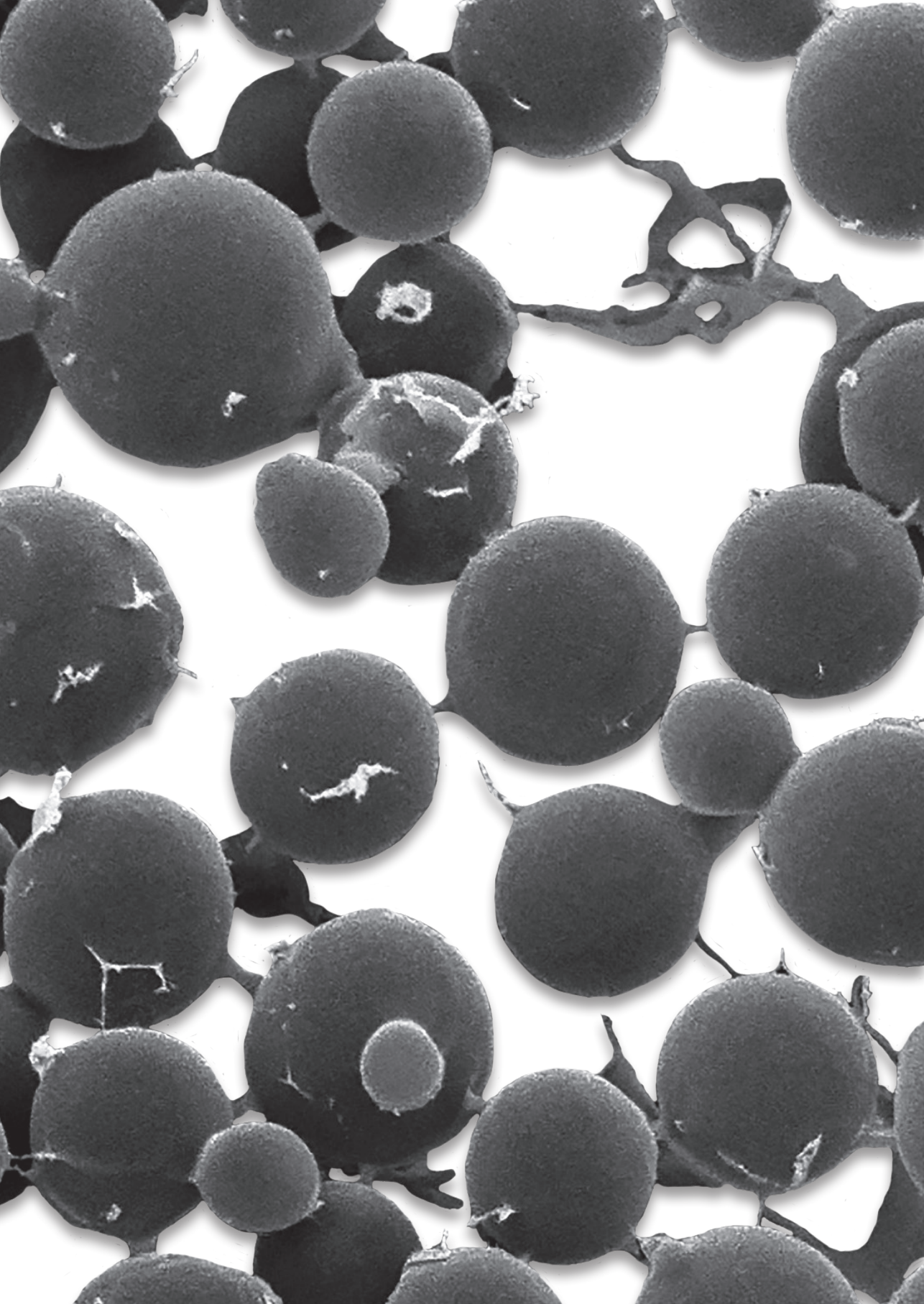
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Chapter 5

Molecular characterization and antifungal susceptibility testing of *Cryptococcus neoformans sensu stricto* from southern Brazil

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Abstract

Cryptococcosis is acquired from the environment by the inhalation of *Cryptococcus* cells and may establish from an asymptomatic latent infection into pneumonia or meningoencephalitis. The genetic diversity of a *Cryptococcus neoformans* species complex has been investigated by several molecular tools, such as multi-locus sequence typing, amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism and microsatellite analysis. This study aimed to investigate the genotype distributions and antifungal susceptibility profiles of *C. neoformans sensu lato* isolates from southern Brazil. We studied 219 *C. neoformans sensu lato* isolates with mating- and serotyping, AFLP fingerprinting, microsatellite typing and antifungal susceptibility testing. Among the isolates, 136 (69%) were from HIV-positive patients. Only *C. neoformans* mating-type **a** and serotype A were observed. AFLP fingerprinting analysis divided the isolates into AFLP1/VNI ($n = 172$; 78.5%), AFLP1A/VNII ($n = 19$; 8.7%), AFLP1B/VNII ($n = 4$; 1.8%) and a new AFLP pattern AFLP1C ($n = 23$; 10.5%). All isolates were susceptible to tested antifungals and no correlation between antifungal susceptibility and genotypes was observed. Through microsatellite analysis, most isolates clustered in a major microsatellite complex and Simpson's diversity index of this population was $D = 0.9856$. The majority of *C. neoformans sensu stricto* infections occurred in HIV-positive patients. *C. neoformans* AFLP1/VNI was the most frequent genotype and all antifungal drugs had high in vitro activity against this species. Microsatellite analyses showed a high genetic diversity within the regional *C. neoformans sensu stricto* population, and correlation between environmental and clinical isolates, as well as a temporal and geographic relationship.

Keywords: Cryptococcosis; *Cryptococcus neoformans*; amplified fragment length polymorphism; microsatellite typing; mating-type; antifungal susceptibility.

Introduction

The basidiomycetous yeast genus *Cryptococcus* is widely distributed in nature, found in soil, avian excreta, tree hollows and decaying wood [1, 2]. Within the genus, the *C. neoformans*/*C. gattii* species complexes are the most important in medical mycology as they are the main etiologic agents of cryptococcosis [3, 4]. *Cryptococcus* infection is acquired from the environment by inhalation of fungal cells and may establish as an asymptomatic latent infection in immunocompetent hosts or cause pneumonia and meningoencephalitis in immunosuppressed patients [4]. This infection is a common complication among solid organ transplant recipients showing high morbidity and mortality (27 %) during the first year of post-transplantation [5]. Among HIV infected patients, 223,100 new cases of cryptococcal meningitis occur annually, resulting in an estimated 181,100 death [6], especially in developing countries, where access to antiretroviral therapy is still limited [7].

The taxonomy of the *Cryptococcus* genus was recently revised [3, 8, 9]. Both *C. neoformans* varieties were raised to species (*C. neoformans sensu stricto* and *C. deneoformans*) and the five *C. gattii* genotypes were grouped into five species (*C. gattii sensu stricto*, *C. bacillisporus*, *C. deuterogattii*, *C. tetragattii* and *C. decagattii*) [3]. This subject has been discussed among the scientific community, with some researchers endorsing the use of 'species complex' instead of using the 'seven recognized species' concept [10]; while others endorse that the different *C. neoformans*/*C. gattii* genotypes need to be considered different species [11].

The genetic diversity of *C. neoformans sensu lato* has been investigated with several molecular tools, such as multilocus sequence typing (MLST) [12–14], amplified length fragment polymorphism (AFLP) fingerprinting [15], restriction fragment length polymorphism (RFLP) [16] and microsatellite analysis [17, 18]. Based on these molecular approaches *C. neoformans s.s.* contains three genotypes AFLP1/VNI, AFLP1A/VNII and AFLP1B/VNII, *C. deneoformans* is genotype AFLP2/VNIV and the hybrid between *C. neoformans s.s.* and *C. deneoformans* is AFLP3/VNIII [3, 15, 19]. Moreover, interspecies hybrids were found between *C. deneoformans* and *C. gattii s.s.* (AFLP2/VNIV×AFLP4/VGI=AFLP8), *C. neoformans s.s.* and *C. gattii s.s.* (AFLP1/VNI×AFLP4/VGI=AFLP9) and *C. neoformans s.s.* and *C. deuterogattii* (AFLP1/VNI_ AFLP6/VGII=AFLP11) [19–21].

Cryptococcus neoformans s.s. genotype AFLP1/VNI is globally the predominant molecular type and causes the majority of cryptococcal infections [22, 23]. *Cryptococcus deneoformans* is geographically restricted to Europe and North

America, and the interspecies hybrid occurs in high rates in Mediterranean Europe, while in other regions it is rarely encountered [15, 19, 22]. In Latin America, *C. neoformans* AFLP1/VNI is the most frequently observed genotype, but the distribution of other genotypes is variable according to the geographic location. Colombia and Brazil have the highest genotypic diverse populations on the South American continent [22, 23]; in addition, some studies have also demonstrated differences in circulating genotypes in each region of Brazil [24–26]. These findings suggest that molecular studies are needed to clarify the genetic diversity and distribution among each microhabitat [27]. Beyond the differences in geographical distribution, studies have suggested that differences in antifungal susceptibilities correlate with species and genotypes [17, 28, 29].

Considering this information, it is necessary to emphasize the importance of investigating epidemiological relationships of clinical and environmental *C. neoformans s.l.* isolates as well as the relationship with antifungal susceptibility profiles. This will allow for a better understanding of the behaviour of this fungus as a saprophyte in the environment and as a pathogen in mammals. This study aimed to investigate the genotypic distribution and antifungal susceptibility profiles of *C. neoformans s.l.* species complex isolates from southern Brazil.

Methods

Isolates and clinical data

For this multicentre study, 219 *C. neoformans s.l.* isolates, previously identified with conventional mycological techniques, were available from mycology laboratories at the Federal University of Paraná, Curitiba, Paraná; Federal University of São Paulo, São Paulo; Federal University of Santa Maria, Santa Maria; Universidade Estadual do Oeste do Paraná (Paraná Western Paraná State University), Cascavel; and Federal University of Uberlândia, Uberlândia, Brazil. In total, 197 originated from clinical, 19 from environmental, two from veterinary samples and one was from an unknown source. The isolates were collected and stored between 1987 and 2015. For clinical isolates, we accessed the patients' medical records to collect clinical information.

Genomic DNA extraction

The isolates were subcultured for 2 days at 30 °C onto Sabouraud dextrose agar for genomic DNA extraction. A loop full of cells was suspended in 300 µl bacterial lysis buffer (Roche Diagnostics, Almere, The Netherlands), followed by mechanical lysis in a MagNA Lyser (Roche Diagnostics) and centrifugation for 2 min at 12.000×g, and

inactivation at 100 °C for 10 min. Then 200 µl of the solution was used for automatic DNA extraction by using the MagNA Pure 96 platform (Roche) with a final elution volume of 100 µl [30].

Mating-type, serotype and genotyping

The mating-type was determined by partial amplification of the RUM1 gene as described before [31]. *Cryptococcus neoformans s.l.* strains 125.91 (=CBS10512; **aA**; AFLP1/VNI), H99 (=CBS8710; **αA**; AFLP1/VNI), JEC20 (=CBS10511; **aD**; AFLP2/VNIV) and JEC21 (=CBS10513; **αD**; AFLP2/VNIV) were included as controls [31]. AFLP fingerprinting analysis was performed as previously described [30]. Raw data was analysed using BioNumerics version 7.5 (Applied Maths, Sint-Martens-Latem, Belgium) with Pearson's correlation coefficient and unweighted pair group method with arithmetic mean clustering algorithm, DNA fragments in the range of 20 bp and to 600 bp were included in the analysis.

Microsatellite typing

The genetic relationship of *C. neoformans s.l.* isolates was investigated by using a nine-marker microsatellite panel as described before [18]. The minimum spanning tree was calculated using BioNumerics version 7.5 (Applied Maths, Sint-Martens-Latem, Belgium) [18].

Antifungal susceptibility testing

Altogether, 109 isolates were randomly selected for antifungal susceptibility testing, which was performed according to CLSI protocol M27-A3 [32]. The antifungal agents tested were amphotericin B (Bristol Myers Squibb, Munich, Germany), fluconazole (Pfizer, Sandwich, UK), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), voriconazole (Pfizer), posaconazole (Merck, NJ, USA), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland) and flucytosine (ICN Pharmaceuticals, Zoetermeer, the Netherlands). The concentration ranges were 0.016 to 16 mg L⁻¹ for amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole, and 0.062 to 64 mg L⁻¹ for fluconazole and flucytosine. *Cryptococcus* isolates were cultured onto Sabouraud dextrose agar for 48 h at 30 °C and the inocula were adjusted to 1 × 10³ – 5 × 10³ CFU mL⁻¹ in 0.9% NaCl to perform the test. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls. MIC was defined as the lowest concentration that produced complete growth inhibition for amphotericin B and a prominent decrease of growth (50 %) for other antifungal agents, compared with the drug-free growth control. Interpretation of MIC values was based on epidemiological cutoff values [33–35].

Data analysis

MIC₅₀ and MIC₉₀ were obtained by ordering the data for each antifungal in ascending order and selecting the median and 90th quantile, respectively. Geometric mean MICs were calculated using Microsoft Office Excel 2010 software. When the MIC was higher or less than the dilutions tested, 1 log₂ dilution higher or 1 log₂ dilution lower was considered to calculate the geometric mean. A two-tailed Mann–Whitney–Wilcoxon non-parametric test was applied to compare the MIC values between different *C. neoformans* genotypes. The test was performed by StatistiXL software, version 1.8 (StatistiXL, Nedland, WA, Australia) and a P value of ≥ 0.05 was considered statistically significant.

Results

Molecular characterization

Among the 219 *C. neoformans sensu lato* isolates, 218 were serotype A and mating-type α by qPCR and one isolate remained undetermined. AFLP fingerprinting analysis divided the isolates into four clusters (Fig. 1). Most of the isolates clustered together with reference strains for *C. neoformans sensu stricto* AFLP1/VNI ($n = 172$; 78.5%). Nineteen isolates (8.7 %) belong to the subgroup AFLP1A/VNB/VNII, and four (1.8%) isolates belong to the minor genotype AFLP1B/VNII. Twenty-three (10.5%) isolates composed a singular cluster AFLP1C, with a new AFLP sub-genotype. One (0.5%) isolate had an AFLP profile not assigned.

Microsatellite genotyping and genetic diversity

When all nine microsatellite CNA markers were combined, 129 different microsatellite genotypes were distinguished among 219 isolates, 93 being unique microsatellite genotypes. Using Simpson's diversity index (D), the discriminatory power for the complete set of nine microsatellite markers was 0.9856.

The relationship between different microsatellite genotypes is illustrated in Fig. 2. There is a major cluster composed of 117 isolates differing from each other by one out of nine microsatellite markers (Fig. 2a). The distribution of clinical, environmental and veterinary isolates is shown in Fig. 2 (a). Overall, 14 out of 19 environmental isolates represent unique microsatellite genotypes but they are close to clinical isolates with only one locus difference from each other, and five of them are grouped in a minor cluster. The composition of two minor clusters is mostly of isolates coming from HIV-positive patients (Fig. 2b). The comparison with AFLP distribution shows that AFLP1A/VNI isolates and AFLP1C are related to each

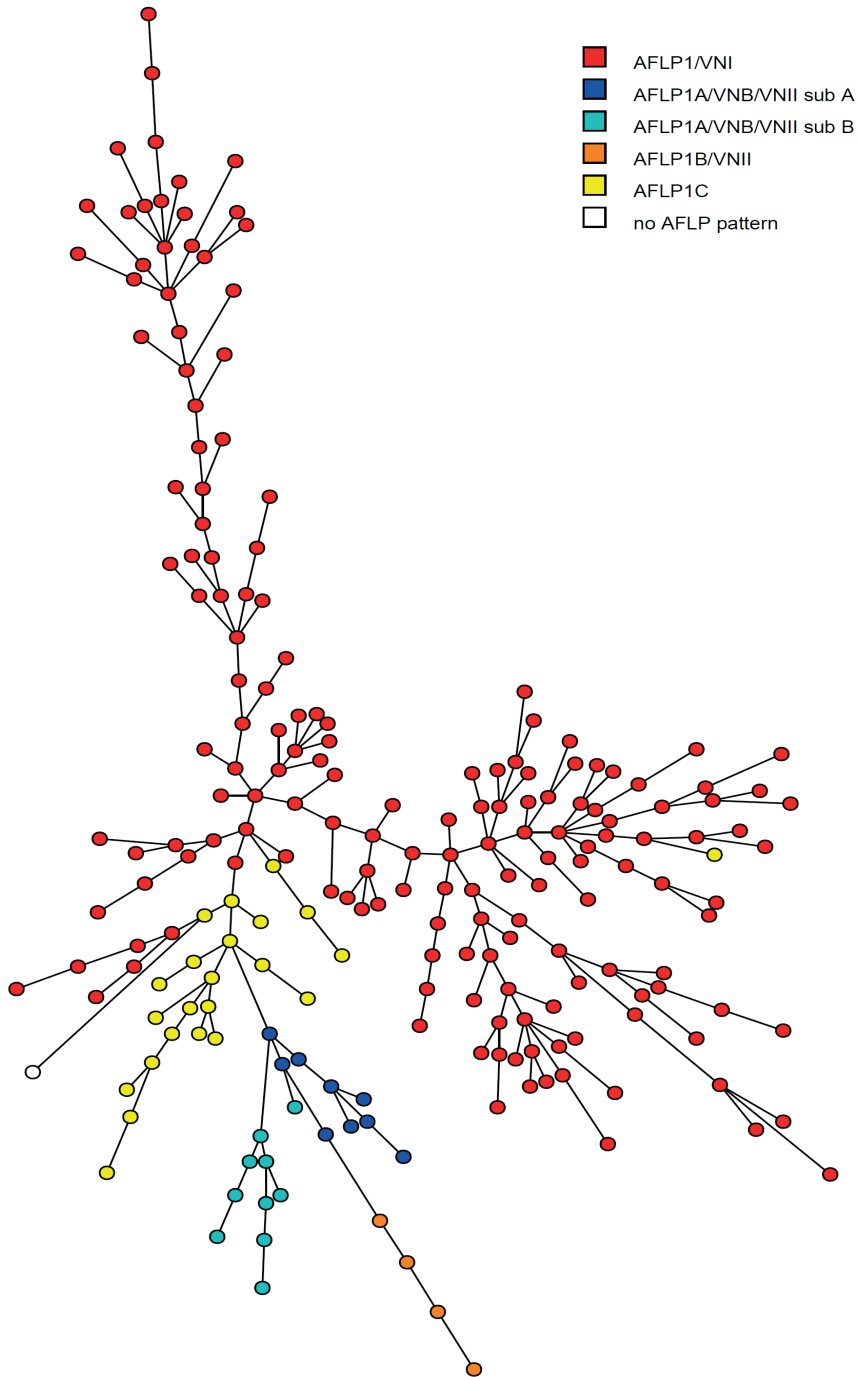


Fig. 1. AFLP minimum spanning tree of 219 Brazilian *C. neoformans sensu stricto* isolates.

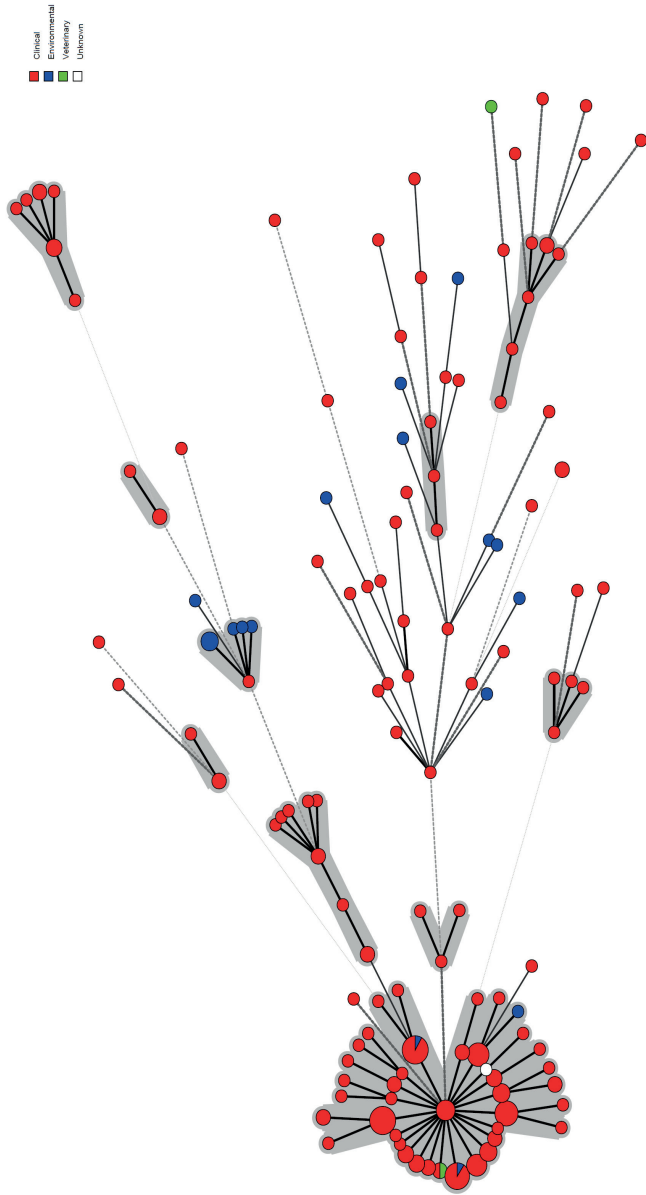


Fig. 2A. Genotypic diversity of *C. neoformans sensu stricto* isolates based on specific microsatellite typing. Minimum spanning tree showing 219 *C. neoformans* s.s. isolates based on a nine-loci microsatellite typing panel. Each circle is a unique microsatellite genotype and the size corresponds to the number of isolates with the same genotype. Connecting lines correspond to the number of differences between genotypes. The solid thick line connects genotypes differing in one locus; the solid thin line connects genotypes different in two–three loci; the dashed line connects genotypes different in four or more loci. (a) Distribution of microsatellite typing according to the source of isolates, clinical, environmental and veterinary. (b) Distribution according to aetiology. (c) Distribution of AFLP genotypes and microsatellite typing. (d) Microsatellite distribution according to year of isolation. (e) Microsatellite distribution according to geographic areas of isolation.

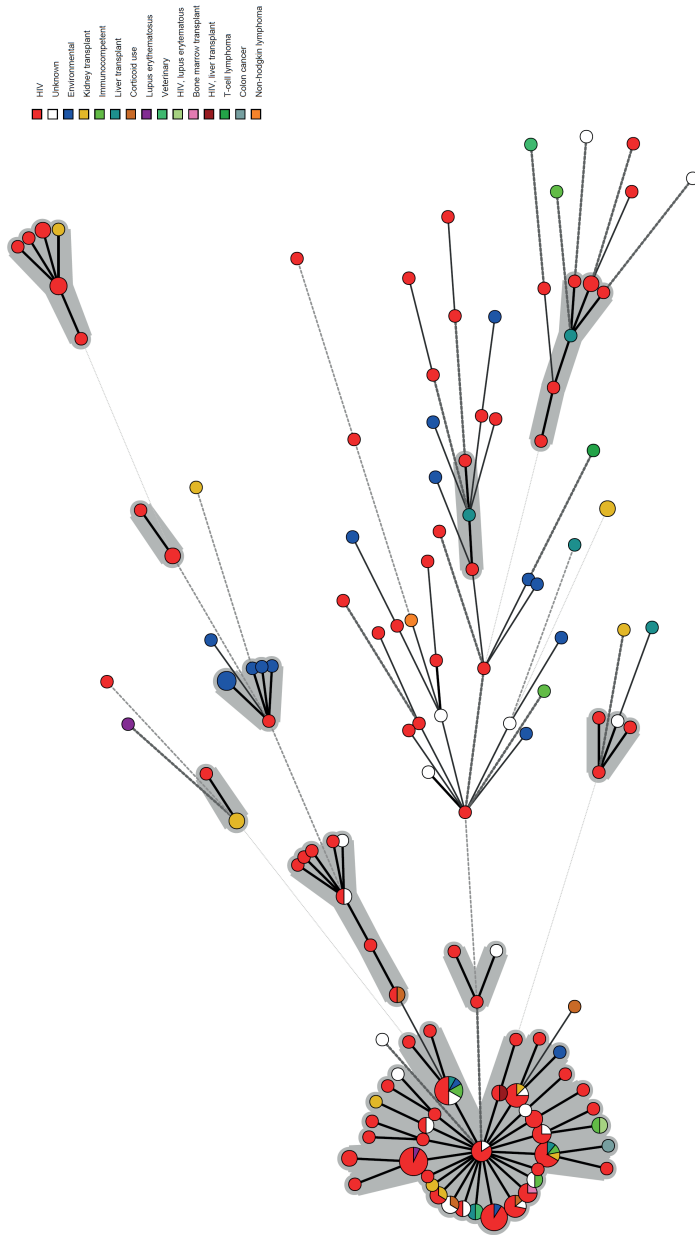


Fig. 2B. Genotypic diversity of *C. neoformans sensu stricto* isolates based on specific microsatellite typing. Minimum spanning tree showing 219 *C. neoformans* s.s. isolates based on a nine-loci microsatellite typing panel. Each circle is a unique microsatellite genotype and the size corresponds to the number of isolates with the same genotype. Connecting lines correspond to the number of differences between genotypes. The solid thick line connects genotypes differing in one locus; the solid thin line connects genotypes different in two–three loci; the dashed line connects genotypes different in four or more loci. (a) Distribution of microsatellite typing according to the source of isolates, clinical, environmental and veterinary. (b) Distribution according to aetiology. (c) Distribution of AFLP genotypes and microsatellite typing. (d) Microsatellite distribution according to year of isolation. (e) Microsatellite distribution according to geographic areas of isolation.

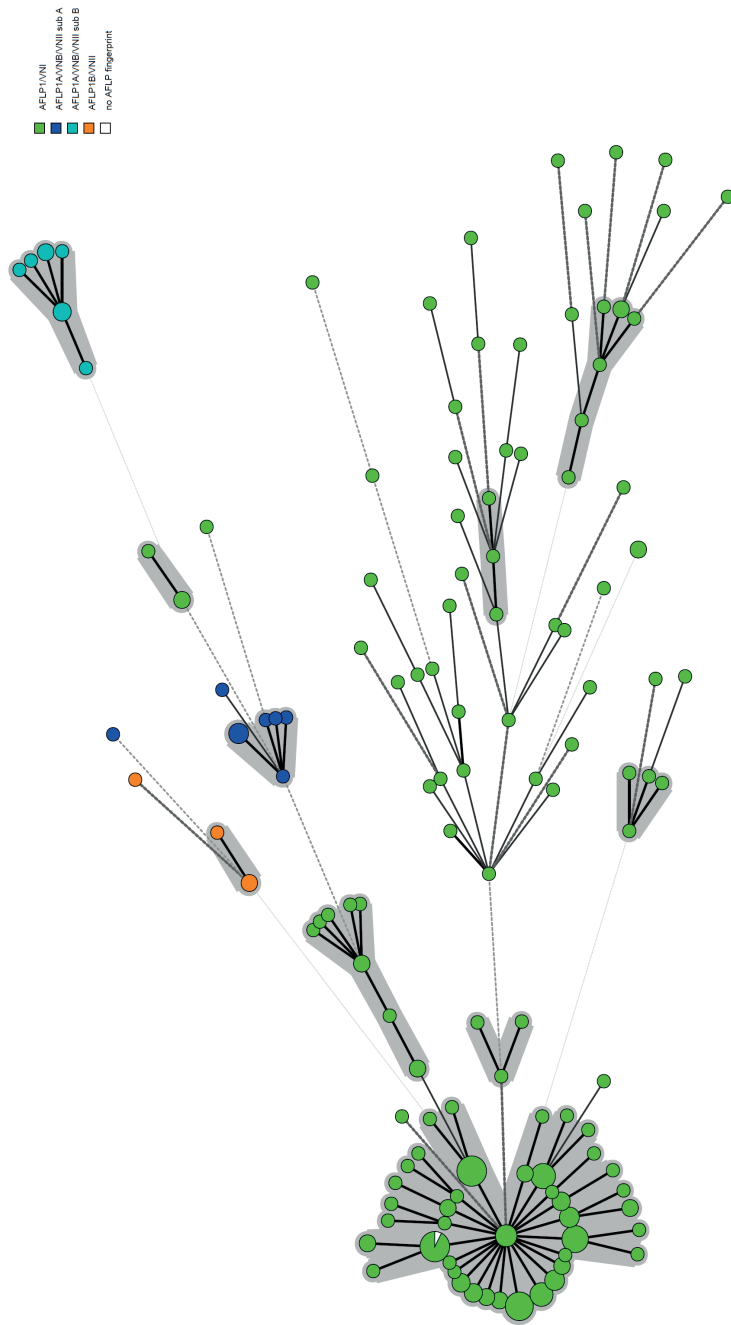


Fig. 2C. Genotypic diversity of *C. neoformans sensu stricto* isolates based on specific microsatellite typing. Minimum spanning tree showing 219 *C. neoformans* s.s. isolates based on a nine-loci microsatellite typing panel. Each circle is a unique microsatellite genotype and the size corresponds to the number of isolates with the same genotype. Connecting lines correspond to the number of differences between genotypes. The solid thick line connects genotypes differing in one locus; the solid thin line connects genotypes different in two–three loci; the dashed line connects genotypes different in four or more loci. (a) Distribution of microsatellite typing according to the source of isolates, clinical, environmental and veterinary. (b) Distribution according to aetiology. (c) Distribution of AFLP genotypes and microsatellite typing. (d) Microsatellite distribution according to year of isolation. (e) Microsatellite distribution according to geographic areas of isolation.



Fig. 2D. Genotypic diversity of *C. neoformans sensu stricto* isolates based on specific microsatellite typing. Minimum spanning tree showing 219 *C. neoformans* s.s. isolates based on a nine-loci microsatellite typing panel. Each circle is a unique microsatellite genotype and the size corresponds to the number of isolates with the same genotype. Connecting lines correspond to the number of differences between genotypes. The solid thick line connects genotypes differing in one locus; the solid thin line connects genotypes different in two–three loci; the dashed line connects genotypes different in four or more loci. (a) Distribution of microsatellite typing according to the source of isolates, clinical, environmental and veterinary. (b) Distribution according to aetiology. (c) Distribution of AFLP genotypes and microsatellite typing. (d) Microsatellite distribution according to year of isolation. (e) Microsatellite distribution according to geographic areas of isolation.

other differing in 0–4 loci (Fig. 2c). Most of the AFLP1A/VNB/VNII sub A isolates combine together in a minor cluster and represent environmental isolates (Fig. 2c). Interestingly, all AFLP1A/VNB/VNII sub B isolates cluster in a minor microsatellite complex and eight out of nine are from HIV-positive patients (Fig. 2c). Temporal distribution shows the presence of the same genotype over prolonged periods, being frequently isolated throughout almost three decades of study (Fig. 2d). When geographic distributions were considered, the isolates from different cities in the current study were highly related to each other, with clusters containing isolates from different locations (Fig. 2e).

Table 1. MIC of *C. neoformans sensu stricto* isolates from southern Brazil

| Species | Antifungal | MIC value ranges (mg L ⁻¹) | | | |
|--|----------------|--|-------------------|-------------------|-------|
| | | Range | MIC ₅₀ | MIC ₉₀ | GM |
| <i>C. neoformans</i> – all genotypes (n=109) | Amphotericin B | <0.016-0.125 | 0.125 | 0.125 | 0.098 |
| | Fluconazole | 0.125-8 | 0.5 | 0.5 | 0.516 |
| | Itraconazole | <0.016-0.25 | 0.031 | 0.063 | 0.027 |
| | Voriconazole | <0.016-0.125 | 0.031 | 0.031 | 0.021 |
| | Posaconazole | <0.016-0.125 | 0.031 | 0.063 | 0.027 |
| | Isavuconazole | <0.016-0.063 | <0.016 | 0.031 | 0.011 |
| | Flucytosine | 0.25-8 | 2 | 4 | 2.42 |
| <i>C. neoformans</i> – AFLP1/VNI (n=99) | Amphotericin B | <0.016-0.125 | 0.125 | 0.125 | 0.099 |
| | Fluconazole | 0.125-8 | 0.5 | 0.5 | 0.521 |
| | Itraconazole | <0.016-0.25 | 0.031 | 0.063 | 0.026 |
| | Voriconazole | <0.016-0.125 | 0.016 | 0.063 | 0.022 |
| | Posaconazole | <0.016-0.125 | 0.031 | 0.063 | 0.027 |
| | Isavuconazole | <0.016-0.063 | <0.016 | 0.031 | 0.011 |
| | Flucytosine | 0.25-8 | 2 | 8 | 2.519 |

GM, geometric mean.

Antifungal susceptibility testing

All isolates showed susceptible MIC values for the antifungal agents tested (Table 1). The new triazoles had high activity against the genotypes tested with MIC ranges of <0.016 – 0.125 mg L⁻¹ for voriconazole and posaconazole and <0.016 – 0.063 mg L⁻¹ for isavuconazole. For fluconazole, two genotype AFLP1/VNI isolates showed a MIC at the limit of wild-type population of 8 mg l⁻¹. We also observed that flucytosine was the less effective antifungal agent, showing the highest geometric mean MIC of 2.42 mg L⁻¹ among the antifungals tested. No significant differences

were observed among the genotypes AFLP1/VNI, AFLP1A/VNII and AFLP1B/VNII and their respective antifungal susceptibility profiles (Mann–Whitney–Wilcoxon’s test, amphotericin B $P = 0.319$; fluconazole $P = 0.386$; itraconazole $P = 0.548$; voriconazole $P = 0.121$; posaconazole $P = 0.092$; isavuconazole $P = 0.431$; flucytosine $P = 0.191$).

Clinical data

Among the clinical isolates, 136 (69%) were isolated from HIV-infected patients and 29 (14.7%) were from patients with another immunosuppressive condition (transplant, $n = 20$; corticosteroid use, $n = 4$; other predisposing factors, $n = 5$). In addition, seven patients (3.6%) had no immunosuppressive condition and 25 (12.7%) had an unknown immune status. The age ranged from 9 to 78 years, with a median of 38 years and the majority of patients were male (65%, $n = 128$).

Discussion

In the current study, 219 *C. neoformans s.l.* isolates from southern Brazil, collected during the period 1987–2015, were molecularly characterized and a randomly chosen subset was subjected to antifungal susceptibility testing. With qPCR sero- and mating-type determination, it was observed that all isolates, except one, were serotype A and mating-type α . Globally, the majority of the clinical and environmental *C. neoformans s.s.* isolates are mating-type α ([17, 22, 30, 36–39], present study). This feature suggests that *C. neoformans s.s.* is a predominantly clonal population with few recombination events and low genetic diversity that allows the maintenance of genomic markers associated with virulence [40, 41]. The presence of both mating-types facilitates sexual reproduction and recombination in a population. However, an exclusively mating-type α population may also have genetic recombination due to the capability of unisexual reproduction [42]. In specific geographic areas, for example India, unisexual reproduction is responsible for recombination and gene flow among *C. neoformans s.s.* populations [43]. In southern Africa mating-type α was found in 12% of *C. neoformans s.s.* clinical isolates obtained from HIV-positive patients [44], and among the clinical isolates from HIV-positive patients from Zimbabwe, 7.3% of *C. neoformans s.s.* was mating-type α [45]. The presence of both mating-types among the African isolates is responsible for the genetic recombination within the *C. neoformans s.s.* population [45, 46].

Cryptococcus neoformans s.s. AFLP1/VNI is the most frequent genotype among clinical and environmental isolates around the world [22, 23]. In Brazil, this genotype has been isolated in 77.5–98% of the clinical and environmental isolates

[24, 26, 47]. This was also observed in the present study where 78.5% ($n = 172$) of *C. neoformans* isolates were genotype AFLP1/VNI. In other countries of Latin America, this genotype also has a high prevalence. In Argentina, all environmental isolates belong to genotype AFLP1/VNI [48, 49]. In Colombia, 97% of the clinical isolates are AFLP1/VNI [50] and 74.6% of *C. neoformans* clinical isolates in Mexico are AFLP1/VNI [51]. In Peru, genotype AFLP1/VNI was found in 50% of the isolates, but genotype AFLP1B/VNII was observed at a frequency of 34.4% [52], different from the present study and other Brazilian studies where genotype AFLP1B/VNII was rarely found [24, 38]. Interestingly, we found 23 isolates which clustered in a clade not described before, representing a new AFLP subgenotype (Fig. 1). The correlation between AFLP genotype and antifungal susceptibility has been discussed during the last few years, and variable data are available in the literature. Some studies have observed a correlation between the genotype and the antifungal susceptibility profile, showing that *C. neoformans* s.s. AFLP1/VNI was found to be less susceptible to amphotericin B, fluconazole, itraconazole and flucytosine when compared to *C. denoformans* AFLP2/VNIV and the interspecies hybrid AFLP3/VNIV [17, 28, 29]. Similar to our results, in the Netherlands, Hagen and colleagues [17] observed less susceptible isolates for flucytosine and fluconazole with MIC ranges of 0.5 – 64 and 0.25 – 16 mg L⁻¹, respectively [17]. In contrast, some studies found no relation between *C. neoformans* AFLP genotypes and antifungal susceptibility [26, 27, 31, 52]. This is in agreement with the present study, where no correlation between subgenotype and antifungal susceptibility was found. Cryptococcosis caused by *C. neoformans* s.s. in Brazil is mainly found in median-aged male patients, diagnosed with HIV or another immunosuppressive condition ([25, 27, 38, 47, 53–55], present study). In agreement with other Latin American countries, in Brazil the majority of clinical *C. neoformans* s.s. isolates are from HIV-positive patients [48, 50, 56].

Microsatellites are tandem repeats present throughout the genome, but highly variable in number and repeat units for each individual within a population [57]. The higher mutation rates of microsatellite loci allow the investigation of intra-population genetic diversity [58]. In studies when closely related isolates need to be differentiated, microsatellite typing may be highly informative [58]. This approach is a high-resolution tool to sub-type *C. neoformans* s.s., providing excellent discrimination between isolates from different sources and allowing epidemiology studies [17, 18, 59]. In our study, we observed a major microsatellite complex composed mainly of clinical isolates that differ from each other by one locus, with only a few environmental and veterinary isolates that were closely related (Fig. 2a). These results differ from Illnait-Zaragozı and colleagues [18], where clinical and environmental isolates were clustered separately, suggesting additional ecological

niches of *C. neoformans s.l.* in the environment and a different relation with hosts in Cuba [18]. However, the authors studied a large number of environmental isolates and our study had a dataset with the majority of isolates with a clinical background. We observed a relation between the microsatellite genotypes and AFLP genotypes (Fig. 2c), showing that each AFLP genotype clustered in different microsatellite clusters. Although both AFLP1A/VNB/VNII subgroups seem to be more genetically isolated, once both were in separated clusters. Temporal and geographic distribution showed that besides the presence of unique microsatellite genotypes, there are isolates from different locations and periods sharing similar microsatellite genotype and clustering together (Fig. 2d, e).

We observed high genetic diversity among the isolates of our dataset, Simpson's diversity index was 0.9856, with 129 different microsatellite genotypes among 219 isolates, 93 being unique. The genetic diversity of southern Brazilian isolates was lower than observed in Cuba ($D = 0.993$) where 104 genotypes were found among 190 isolates and also lower than observed in the Netherlands ($D = 0.994$) where 196 genotypes were found among 259 isolates [17, 18]. In an Asian study, 265 different genotypes were found among 493 isolates and the genetic diversity varied according to the country. Kuwait and Qatar showed the most diversity in the population ($D = 1.000$), followed by Japan ($D = 0.998$), Indonesia ($D = 0.994$), India ($D = 0.983$), China ($D = 0.975$) and Thailand ($D = 0.968$). The authors also observed a relation between antifungal-resistant profiles in three specific microsatellite complexes (MC2, MC3 and MC17) [59], which was not observed in the present study. In Brazil, Ferreira-Paim and colleagues [54], using the ISHAM consensus MLST scheme, found a highly clonal population structure of *C. neoformans s.s.* in southeastern Brazil, and less variability when compared to Africa [54]. In contrast, our data showed high genetic diversity among *C. neoformans s.s.* isolates. However, different molecular approaches were used to analyse the genetic variability in both studies. MLST shows the polymorphisms in nucleotide sequences and allows the construction of a database; however, MLST fails to detect variability when the population has insufficient genetic variation, as occurs among individuals from a recent speciation [60]. Chen and colleagues [46] using MLST analysed the population structure of *C. neoformans s.s.* from Botswana and found evidence of a clonal network by eBURST analysis; however, STRUCTURE and SplitsTree analyses showed recombination events between the genotypes VNI and VNB [46]. A similar discrepancy was also observed by Nyazika and colleagues using microsatellite typing of *C. neoformans s.s.* from Zimbabwe [45]. Contradictory results were obtained from the same dataset, where disequilibrium linkage tests were significant, but Simpson's diversity index showed a high genetic diversity.

In conclusion, genotype AFLP1/VNI was the most frequent among the isolates studied and all *C. neoformans sensu stricto* isolates had low MICs of antifungal drugs. Microsatellite analysis revealed high diversity among the *C. neoformans sensu stricto* studied population with clinical, environmental and veterinary isolates being related to each other. In addition, the isolates showed a temporal and geographic relationship.

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Conflicts of interest

J.F.M. served as consultant to and has received research grants from Astellas, Basilea, Gilead Sciences and Merck. All of the other authors declare no conflicts of interest.

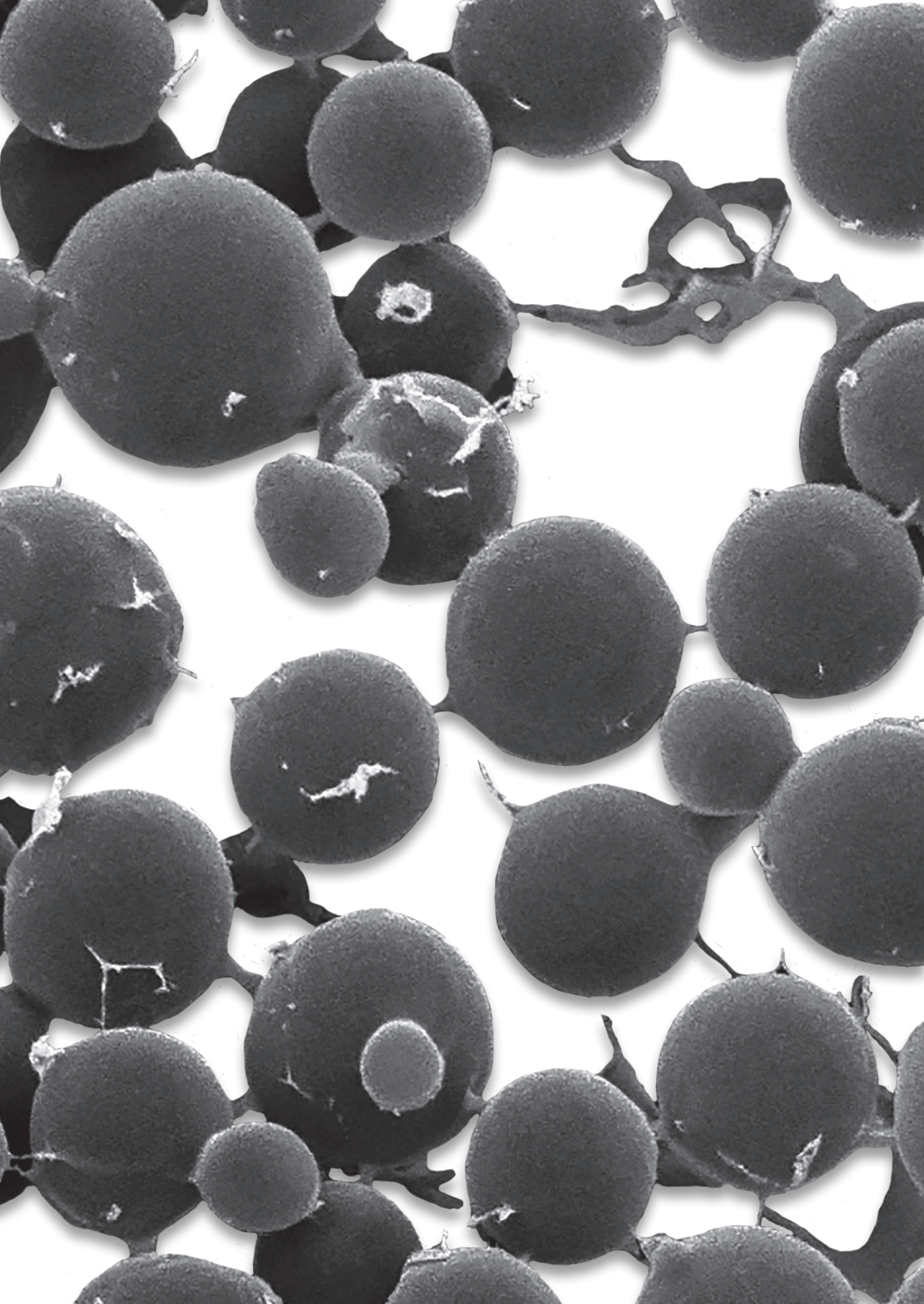
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Chapter 6

Summary and general discussion

Samenvatting en discussie

Resumo e discussão geral

Summary and general discussion

Epidemiological studies are important to recognize environmental reservoirs of *Cryptococcus* species and are sentinels for the appearance of different genotypes in the environment. The knowledge of circulating species and genotypes in a particular region where molecular diagnosis is not available it is necessary to predict the probable etiological agent of cryptococcosis cases. In addition, determination of the antifungal susceptibility allows the detection of non wild-type isolates and may help to choose a better therapy for these specific cases.

Chapter 2 is a review about worldwide ecological niches of *C. gattii s.l.*, focusing on the ecoepidemiology of the *C. gattii* species complex in developing countries. We observed that *C. gattii s.l.* are distributed in a plethora of ecological niches and each year new reservoirs are discovered around the World. However, many countries have no epidemiological data available about *Cryptococcus* spp., clinical frequency or environmental distribution. In Brazil, cryptococcal epidemiologic information is limited, there being many regions unaware about the local epidemiology [1]. The majority of published data are from the Brazilian Southeast region, with few information from South, Midwest, Northeast and North regions [2-9]. The most frequently isolated species of environmental and clinical samples in Brazil are *C. deuterogattii* and *C. neoformans s.s.* [10]. Cryptococcosis caused by *C. gattii s.s.* and *C. bacillisporus* also occur in the South, Southeast and Northeast of Brazil.

In **chapter 3** it was observed that the studied isolates of *C. deuterogattii* belonged to mating-types **a** and **α**. The presence of both mating-types in nearly equal quantities may be related to recombination events among individuals of a population, being responsible for the genetic diversity observed among *C. deuterogattii* isolates [11]. However, most of the isolates from this study belonged to mating-type **a** therefore, recombination analyses should be performed to verify if this population has predominantly clonal or recombinant expansion. AFLP analysis showed the presence of two distinct clades representing new AFLP genotypes that were separated due to the presence of dominant markers. To identify which genetic markers made-up these new AFLP genotypes and its biological aspects of *C. deuterogattii*, Sequence Characterized AFLP Regions Multi-Locus Typing (SCAR-MLST) may be performed to verify the genomic locations of dominant markers and their influence on virulence traits, such as melanin and capsule production. A clinical isolate of *C. deuterogattii* (UFU986) was genetically indistinguishable from the highly virulent AFLP6A/VGIIa genotype that was responsible for a cryptococcosis outbreak on Vancouver Island, Canada (see Figure 1 in Chapter 3). This isolate was obtained in 2008 from a cerebral

spinal fluid sample of an apparently immunocompetent male patient who suffered from systemic hypertension as the sole medical history. In vitro susceptibility tests have shown that UFU986 is wild type for the antifungal tested. Virulence assays can be performed to investigate whether the virulence of UFU986 is comparable to the outbreak lineage of Vancouver Island [12]. In addition, the isolates G14 and G18 were obtained from immunocompetent patients, from the same city, and both developed pulmonary and central nervous system infections that were cured but remained with sequelae (see Figure 2, chapter 3). The first one (G14, mating-type **a**) was from a male patient who worked as a lumberjack at the time when cryptococcosis started; the second (G18, mating-type **a**) was a female patient who worked as a repository in a supermarket and reported constant contact with wooden boxes. We speculate that both patients may have been infected by *C. deuterogattii* during work activities, since they had constant contact with wood products which is the main environmental niche of the *C. gattii* species complex.

Species belonging to the *C. gattii* species complex vary in capsule and cell size, thermotolerance, geographic distribution and some species affect immunocompetent patients while others affect immunocompromised patients [13-15]. Results in **chapter 4** show that the species have also different abilities to induce cytokines and antimicrobial molecules in human peripheral blood mononuclear cells (PBMCs). We observed that *C. bacillisporus* and *C. deuterogattii* induced the lowest levels of tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and IL-6 among the five species of the *C. gattii* complex. *Cryptococcus deuterogattii* induced higher levels of IL-22 than *C. tetragattii*. In addition, *C. bacillisporus* and *C. gattii s.s.* showed the ability to proliferate inside human monocyte-derived macrophages after 24 h of in vitro infection. All *C. gattii s.l.* species were able to generate reactive oxygen species (ROS) in human PBMCs, although *C. bacillisporus* and *C. deuterogattii* were more efficient than the other species. Overall, *C. bacillisporus* and *C. deuterogattii* induced lower levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 and higher ROS levels than those induced by other species. Interestingly, Li et al. [16] investigated the association between human genetic variability and cytokine production in response to several bacterial, fungal, and viral stimuli. These authors found a single nucleotide polymorphism (SNP-rs4496335) related to TNF- α levels and with moderate association with IL-1 β and IL-6 levels in response to *C. deuterogattii* (called *Cryptococcus* in the original article), this SNP was also related to IL1RN gene expression. To confirm these results, the authors showed that the preincubation of PBMCs with the IL-1 receptor antagonist (IL-1Ra) inhibits the induction of TNF- α by *C. deuterogattii* [16]. The ability to cause disease in immunocompetent and

immunocompromised humans may be dependent on both the pathogen and host genetic characteristics [17-19]. Three isolates of each species of the *C. gattii* complex were tested and the differences observed after stimulation of PBMCs were dependent on the species and cytokine evaluated (data not shown). For *C. gattii* s.s. there was a significant difference between the isolates only in IL-10 production and for *C. bacillisporus* a significant difference was observed in IL-1 β production. Genetic variability of the host is an important factor in the determination of disease processes, so additional studies focused on the investigation of the genomic or epigenetic profile may help to clarify how host variability and pathogen variability contribute to the establishment of diseases.

In **chapter 5** we observed that all isolates of *C. neoformans* s.l. were mating-type α and serotype A, most of them ($n = 172$; 78.5%) clustered with the AFLP1/VNI reference strain and 23 isolates (10.5%) composed a new AFLP genotype named here as AFLP1C. Interestingly, The AFLP1A sub B genotype was grouped into a smaller microsatellite cluster (MC) and 8 of 9 isolates from this cluster were from HIV-positive patients. Pan and colleagues [20] observed that most isolates of a specific microsatellite genotype, called MC8, were associated with HIV-positive patients, and MC17 had the majority of non-wild isolates for flucytosine. A relation between microsatellite and antifungal susceptibility was not observed in the present study. Microsatellite analyses revealed 129 different genotypes in a dataset of 219 isolates, with 93 unique genotypes. Most isolates ($n = 117$) clustered in a major MC differing from each other by 1 out of 9 microsatellite markers (see Figure 2a, chapter 5). Simpson's diversity index showed a high genetic diversity ($D = 0.9856$) among Southern Brazilian *C. neoformans* s.s. isolates. In addition, we observed that many *C. neoformans* s.s. genotypes were isolated from different geographic regions throughout the three decades of study, suggesting that these genotypes are permanently settled in the environment. Clinical and environmental *C. neoformans* s.s. isolates were genetically related, differing from each other in one microsatellite locus. The same relationship was observed for the *C. neoformans* s.s. populations from Japan and Thailand [20]. In contrast, an epidemiological study conducted in Cuba revealed that clinical and environmental isolates of *C. neoformans* s.s. were grouped into different MCs, showing different environmental niches for this genotype [21]. A limitation of the present study was the presence of only 19 environmental isolates. Future experiments have to address an environmental sampling to include more isolates of *C. neoformans* s.s. in the microsatellite analysis.

In vitro antifungal susceptibility tests showed that *C. neoformans* and *C. gattii* species complex vary in their susceptibility, with higher MIC values for the *C. gattii* species complex compared to the *C. neoformans* species complex [22-25]. In addition, differences in antifungal susceptibility were found also inside the complexes, with *C. deuterogattii* (AFLP6/VGII) and *C. neoformans* s.s. (AFLP1/VNI) showing less susceptibility to azoles [26-28] when compared to other species inside these complexes. *In vitro* antifungal susceptibility tests performed in **chapters 3** and **5** showed that *C. deuterogattii* and *C. neoformans* s.s. isolates were wild-type to all antifungals tested. However, *C. neoformans* s.s. isolates showed high MICs to fluconazole and flucytosine. *In vitro* susceptibility may not predict response to *in vivo* treatment, therefore therapy for cryptococcosis is generally chosen according to the patient's clinical manifestation and immune status, rather than considering the species involved in the infection [29]. However, susceptibility testing is useful for detecting special cases of resistance and thus assisting in the adoption of the best antifungal therapy.

As future prospects, SCAR-MLST analysis with *C. deuterogattii* isolates can be performed to verify the genomic location of the dominant markers of the new AFLP patterns in order to investigate whether these markers affect biological aspects of the fungus, such as virulence. On the other hand, virulence assays may be performed to investigate whether the virulence of UFU986 is comparable to the Vancouver Island outbreak lineage. In addition, further studies should focus on the investigation of the presence of specific SNPs related to susceptibility to cryptococcosis in order to verify how genetic variability of host and pathogen contribute to the establishment of the disease by different *Cryptococcus* species. Furthermore, the environmental isolation of *C. neoformans* s.s. should be carried out with the aim of including a larger number of environmental isolates in microsatellite analyses and thus obtain more robust information on the genetic relationship between the clinical and environmental isolates of *C. neoformans* s.s. in the studied region.

Samenvatting en Discussie

Epidemiologisch onderzoek is belangrijk om omgevingsbronnen van *Cryptococcus*-soorten te herkennen en zijn markers voor het verschijnen van verschillende genotypen in de omgeving. De kennis van circulerende soorten en genotypen in een bepaalde geografische regio, waar geen moleculaire diagnose beschikbaar is, is noodzakelijk om de vermoedelijke verwekker van cryptokokkose te voorspellen. Bovendien maakt bepaling van de antischimmelgevoeligheid de detectie van niet-wild type isolaten mogelijk en kan het helpen om een betere therapie voor deze specifieke gevallen te kiezen.

Hoofdstuk 2 is een samenvatting van de wereldwijde ecologische niches van *C. gattii s.l.*, met de nadruk op de epidemiologie van het *C. gattii* soortcomplex in ontwikkelingslanden. We hebben waargenomen dat *C. gattii s.l.* aanwezig is in een overvloed aan ecologische niches en jaarlijks worden wereldwijd nieuwe reservoirs ontdekt. Veel landen hebben echter geen epidemiologische gegevens beschikbaar over aanwezige *Cryptococcus* soorten, klinische frequentie of verspreiding in de omgeving. In Brazilië is epidemiologische informatie over cryptokokken beperkt, en in veel regio's is de lokale epidemiologie onbekend [1]. De meeste gepubliceerde gegevens zijn afkomstig uit de Braziliaanse regio Zuid-Oost, met weinig informatie uit de regio's Zuid, Midden-Westen, Noord-Oosten en Noorden [2-9]. De meest voorkomende soorten, geïsoleerd uit klinische en omgevingsmonsters in Brazilië zijn *C. deuterogattii* en *C. neoformans s.s.* [10]. Cryptokokkose door *C. gattii s.s.* en *C. bacillisporus* is ook waargenomen in de Zuid-, Zuidoost- en Noordoostelijke-regio's van Brazilië [10].

In **hoofdstuk 3** beschrijven we het voorkomen van *C. deuterogattii* van de mating types α en alfa in Brazilië. De aanwezigheid van beide mating typen in vrijwel gelijke hoeveelheden kan verband houden met recombinaties tussen individuen in een populatie, die verantwoordelijk zijn voor de genetische diversiteit die wordt waargenomen bij *C. deuterogattii*-isolaten [11]. De meeste isolaten in deze studie waren echter van het mating type α . Daarom zouden recombinatieanalyses moeten worden gedaan om te controleren of deze populatie een overwegend klonale of recombinante expansie vertoont. AFLP-analyse toonde de aanwezigheid aan van twee verschillende clades met nieuwe AFLP-genotypen die werden gescheiden door de aanwezigheid van dominante markers. Om te onderzoeken welke genetische markers deze nieuwe AFLP-genotypes bij *C. deuterogattii* hebben gevormd zouden sequentieel gekarakteriseerde AFLP-gebieden met multi-locus-

typering (SCAR-MLST) kunnen worden uitgevoerd. Zo kunnen de genom locaties van dominante markers en hun invloed op virulentie eigenschappen worden geverifieerd, zoals de vorming van melanine en capsules. Een Braziliaans klinisch *C. deuterogattii* isolaat (UFU986) bleek genetisch niet te onderscheiden te zijn van het zeer virulente uitbraak genotype AFLP6A/VGIIa op Vancouver Island, Canada (zie Figuur 1 in hoofdstuk 3). Dit isolaat werd in 2008 geïsoleerd uit hersenvocht van een schijnbaar immunocompetente mannelijke patiënt zonder onderliggend lijden met alleen hypertensie. In vitro gevoeligheidstesten hebben aangetoond dat UFU986 een wilde type gevoeligheidspatroon liet zien. Virulentietesten zouden kunnen worden uitgevoerd om te onderzoeken of de virulentie van UFU986 vergelijkbaar is met de Vancouver Island uitbraak isolaten [12]. Bovendien werden ook de isolaten G14 en G18 verkregen van immunocompetente patiënten, met lastig te genezen long- en centraal zenuwstelsel infecties uit dezelfde stad. (zie figuur 2, hoofdstuk 3). De eerste stam (G14, mating type a), was afkomstig van een mannelijke patiënt die als houthakker werkte; de tweede (G18, mating type α) was een vrouwelijke patiënt die werkte in een supermarkt, waar ze veel contact had met houten kisten. We speculeren dat beide patiënten tijdens hun werk werden geïnfecteerd met *C. deuterogattii*, omdat ze voortdurend in contact zijn met houten producten, de plaats waar het *C. gattii* soortcomplex vaak wordt aangetroffen.

Soorten die behoren tot het *C. gattii* soortcomplex variëren in capsulevorm en celgrootte, thermotolerantie, geografische spreiding en sommige soorten kunnen immunocompetente patiënten infecteren [13-15]. De resultaten gepresenteerd in **hoofdstuk 4** laten ook zien dat sommige soorten verschillen in hun vermogen om cytokines en antimicrobiële moleculen te induceren door mononucleaire cellen verkregen uit perifeer bloed van vrijwilligers (PBMC's). We hebben waargenomen dat *C. bacillisporus* en *C. deuterogattii* het minste de PBMCs aanzette tot productie van tumornecrosefactor-alfa (TNF- α), interleukine-1 β (IL-1 β) en IL-6 vergeleken met de vijf soorten van het *C. gattii* soortcomplex. *C. deuterogattii* induceerde hogere niveaus van IL-22 dan *C. tetragattii*. Bovendien toonden *C. bacillisporus* en *C. gattii* s. s. het vermogen om te prolifereren in humane, van monocyt-afkomstige macrofagen na 24 uren infectie in vitro. Alle *C. gattii* s.l. soorten waren in staat om reactieve zuurstofradicalen (ROS) te genereren PBMC's, hoewel *C. bacillisporus* en *C. deuterogattii* efficiënter waren dan de andere soorten. Over het algemeen induceerden *C. bacillisporus* en *C. deuterogattii* lagere niveaus van de pro-inflammatoire cytokinen TNF- α , IL-1 β en IL-6 en hogere ROS-spiegels dan andere soorten. Li en collega's onderzochten het verband tussen humane genetische variabiliteit en cytokineproductie na blootstelling aan verschillende bacteriële, schimmel- en virale stimuli hebben [16]. Deze auteurs vonden een enkel nucleotide

polymorfisme (SNP-rs4496335) gerelateerd aan de hoogtes van TNF- α waardes en een matige verband met IL-1, 1 β en IL-6 waardes na stimulatie met *C. deuterogattii* (genaamd *Cryptococcus* in het originele artikel). Deze SNP was ook gerelateerd aan de expressie van het IL1RN-gen. Om deze bevindingen te bevestigen, toonden de auteurs aan dat pre-incubatie van PBMC's met de IL-1-receptorantagonist (IL-1Ra) de inductie van TNF- α door *C. deuterogattii* remt [16]. Het vermogen van verschillende *Cryptococcus* soorten om ziekten te veroorzaken bij immuuncompetente en immuungecompromiteerde mensen kan afhankelijk zijn van inherente eigenschappen van de pathogenen en gastheren [17-19]. We testten drie stammen van elke soort in het *C. gattii* soortcomplex en de verschillen na stimulering door PBMC's bleken afhankelijk te zijn van de species en type cytokine dat werd geëvalueerd. Voor *C. gattii* s.s. was er alleen een significant verschil tussen stammen bij de IL-10-productie en voor *C. bacillisporus* was er een significant verschil in IL-1 β -productie. Genetische variabiliteit tussen patiënten is een belangrijke factor bij het bepalen van ziekteprocessen en verdere studies gericht op onderzoek naar genomische of epigenetische profielen kunnen helpen om duidelijk te maken hoeveel variabiliteit van de gastheer en hoeveel van pathogenen bijdraagt tot het ontstaan van cryptokokkose.

In **hoofdstuk 5** wordt beschreven dat alle onderzochte Braziliaanse isolaten mating type a en serotype A waren, waarvan de meeste ($n = 172$; 78,5%) geclusterd waren met de AFLP1/VNI-referentiestam en 23 isolaten (10,5%) een nieuw AFLP-genotype lieten zien, AFLP1C. Interessant was dat AFLP1A subgroep B zich bevond in een klein microsatellietcluster (MC) en dat 8 van de 9 isolaten afkomstig waren van HIV-positieve patiënten. Pan en collega's [20] zagen eerder dat de meeste isolaten van MC8 geassocieerd bleken te zijn met HIV-positieve patiënten en MC17 de meeste niet-wildtype gevoelige isolaten had voor flucytosine. Een verband tussen microsatelliet genotype en antifungale gevoeligheid werd niet waargenomen in ons onderzoek. Microsatelliet analyses onthulden 129 verschillende genotypen in een dataset van 219 isolaten, met 93 unieke genotypen. De meeste isolaten ($n = 117$), geclusterd in een groot MC complex verschilden van elkaar in slechts 1 van de 9 microsatellietmarkers (zie Figuur 2a, hoofdstuk 5). De Simpson diversiteitsindex toonde een hoge genetische diversiteit ($D = 0,9856$) bij de Zuid-Braziliaanse *C. neoformans* s.s. isolaten. Bovendien stelden we vast dat veel *C. neoformans* s.s. genotypen afkomstig waren uit verschillende geografische regio's gedurende de drie decennia van onderzoek, hetgeen suggereert dat deze genotypes permanent in het milieu aanwezig zijn. Klinische en omgevings *C. neoformans* s.s. isolaten waren genetisch gerelateerd, met maximaal slechts verschillend voor één microsatelliet locus. Dezelfde relatie werd waargenomen voor *C. neoformans*

s.s. populaties uit Japan en Thailand [20]. Deze resultaten verschilden met epidemiologisch onderzoek uit Cuba, waar klinische en omgevingsisolaten in verschillende MC's bleken te zijn geclusterd, met verschillende milieu-niches voor de genotypen [21]. Een beperking van de huidige studie was de analyse van slechts 19 omgevingsisolaten. Toekomstige microsatellietanalyse studies moeten meer *C. neoformans* s.s. omgevingsisolaten includeren.

In vitro antifungale gevoeligheidstests hebben aangetoond dat de complexen van *C. neoformans* en *C. gattii*-soorten variëren in gevoeligheid, met hogere MIC-waarden voor het *C. gattii* species complex in vergelijking met het *C. neoformans* species complex [22-25]. Bovendien werden verschillen in antischimmelgevoeligheid ook binnen de complexen gevonden, waarbij *C. deuterogattii* (AFLP6/VGII) en *C. neoformans* s.s. (AFLP1/VNI) de laagste gevoeligheid voor azolen lieten zien [26-28] in vergelijking met andere soorten in deze complexen. In vitro antifungale gevoeligheidstests beschreven in **hoofdstuk 3 en 5** toonden aan dat *C. deuterogattii* en *C. neoformans* s.s. isolaten een wildtype patroon lieten zien voor alle geteste antischimmelmiddelen. *C. neoformans* s.s. isolaten hadden hoge MIC's voor fluconazol en flucytosine. In vitro gevoeligheid kan de respons op behandeling in vivo niet voorspellen, daarom wordt de therapie van cryptokokkose over het algemeen meer gekozen op basis van klinische manifestatie en immuunstatus van de patiënt, dan op basis van de soort die bij de infectie betrokken [29].

In de toekomst kan SCAR-MLST-analyse met *C. deuterogattii* isolaten worden uitgevoerd om de genomische locatie van dominante markers van de nieuwe AFLP-patronen te verifiëren en te onderzoeken of deze de biologische aspecten van de schimmel beïnvloeden zoals virulentie. Daarnaast moeten verdere studies gericht zijn op onderzoek naar de aanwezigheid van specifieke SNP's gerelateerd aan gevoeligheid voor cryptokokkose om na te gaan hoe de genetische variabiliteit van gastheer en pathogeen bijdragen aan het ontstaan van ziekte door verschillende *Cryptococcus* soorten. Tot slot is het belangrijk om meer niet-klinische *C. neoformans* s.s. uit het milieu in de microsatellietanalyses op te nemen en zo meer robuuste informatie te verkrijgen over de genetische relatie tussen klinische en omgevingsisolaten van *C. neoformans* s.s. in Zuid Brazilië.

Resumo e discussão geral

Estudos epidemiológicos são importantes para identificar os reservatórios ambientais das espécies de *Cryptococcus* e são úteis como sentinelas para o aparecimento de diferentes genótipos no ambiente. O conhecimento das espécies e genótipos circulantes em uma região onde o diagnóstico molecular não está disponível é necessário para prever o provável agente etiológico dos casos de criptococose. E ainda, a determinação da suscetibilidade aos antifúngicos permite a detecção de isolados do tipo não selvagem e auxilia na escolha da melhor terapia destes casos específicos.

O **capítulo 2** é uma revisão sobre os nichos ecológicos de *C. gattii s.l.*, focando na epidemiologia das espécies de *C. gattii* em países em desenvolvimento. Observou-se que *C. gattii s.l.* estão distribuídos em uma ampla variedade de nichos ecológicos e que a cada ano novos reservatórios são descobertos ao redor do mundo. Contudo, muitos países não possuem dados epidemiológicos disponíveis sobre a frequência clínica de *Cryptococcus spp.* ou a distribuição ambiental. No Brasil, as informações sobre a epidemiologia de *Cryptococcus spp.* é limitada, com muitas regiões desconhecendo a própria epidemiologia [1]. A maioria dos dados publicados são da região Sudeste do Brasil e há poucas informações disponíveis das regiões Sul, Centro-Oeste, Nordeste e Norte [2-9]. As espécies mais frequentemente isoladas de amostras ambientais e clínicas no Brasil são *C. deuterogattii* e *C. neoformans s.s.* [10]. Criptococose causada por *C. gattii s.s.* e *C. bacillisporus* também ocorrem nas regiões Sul, Sudeste e Nordeste do Brasil [10].

No **capítulo 3** observou-se que os isolados estudados de *C. deuterogattii* pertenciam aos *mating-types* **a** e **α**. A presença de ambos os *mating-types* em proporções semelhantes está relacionada com eventos de recombinação entre os indivíduos de uma população de *C. deuterogattii*, o que pode explicar a diversidade genética observada entre os isolados de *C. deuterogattii* [11]. Contudo, a maioria dos isolados deste trabalho pertenciam ao *mating-type* **α** por isso, análises de recombinação devem ser realizadas para verificar se esta população tem expansão predominantemente clonal ou recombinante. Análises de AFLP mostraram a presença de dois clados distintos representando novos genótipos de AFLP que foram separados devido a presença de marcadores dominantes. Para identificar quais marcadores genéticos compõem esses novos genótipos de AFLP e seus aspectos biológicos em *C. deuterogattii*, análises de *Sequence Characterized AFLP Regions Multi-Locus Typing* (SCAR-MLST) podem ser realizadas para verificar as localizações

genômicas de marcadores dominantes e sua influência sobre características de virulência como a produção de melanina e a produção de cápsula. Um isolado clínico de *C. deuterogattii* (UFU986) foi geneticamente indistinguível do genótipo altamente virulento AFLP6A/VGIIa que foi responsável por um surto de criptococose na Ilha de Vancouver, Canadá (ver Figura 1 no Capítulo 3). Este isolado foi obtido em 2008 a partir de uma amostra de líquido de um paciente do gênero masculino aparentemente imunocompetente que sofria de hipertensão sistêmica como a única doença de base. Testes de suscetibilidade *in vitro* mostraram que o UFU986 é do tipo selvagem para os antifúngicos testados. Ensaios de virulência podem ser realizados para investigar se a virulência do UFU986 é comparável à linhagem do surto da ilha de Vancouver [12]. Além disso, os isolados G14 e G18 foram obtidos de pacientes imunocompetentes, da mesma cidade, e ambos desenvolveram infecções pulmonares e do sistema nervoso central que foram curadas, mas permaneceram com sequelas (ver Figura 2, capítulo 3). O primeiro isolado (G14, *mating-type a*) era de um paciente do gênero masculino que trabalhava como lenhador no momento em que a criptococose iniciou; o segundo (G18, *mating-type a*) era de uma paciente do gênero feminino que trabalhava como repositora em um supermercado e relatou constante contato com caixas de madeira. Nós especulamos que ambos os pacientes podem ter sido infectados por *C. deuterogattii* durante as atividades laborais, já que eles tiveram constante contato com produtos de madeira que é o principal nicho ambiental do complexo de espécies *C. gattii*.

Espécies pertencentes ao complexo de espécies *C. gattii* variam quanto ao tamanho da cápsula e célula, termotolerância, distribuição geográfica e, ainda, algumas espécies afetam pacientes imunocompetentes enquanto outras afetam pacientes imunocomprometidos [13-15]. Os resultados do **capítulo 4** mostram que as espécies do complexo também têm diferentes habilidades para induzir citocinas e moléculas antimicrobianas em células mononucleares do sangue periférico humano (PBMCs). Observou-se que *C. bacillisporus* e *C. deuterogattii* induziram os menores níveis de fator de necrose tumoral alfa (TNF- α), interleucina-1 β (IL-1 β) e IL-6 entre as cinco espécies do complexo *C. gattii*. *Cryptococcus deuterogattii* induziu níveis mais elevados de IL-22 do que *C. tetragattii*. Além disso, *C. bacillisporus* e *C. gattii* s.s. mostraram a capacidade de proliferar dentro de macrófagos humanos derivados de monócitos após 24 h de infecção *in vitro*. Todas as espécies *C. gattii* s.l. foram capazes de gerar espécies reativas de oxigênio (ERO) em PBMCs humanas, embora *C. bacillisporus* e *C. deuterogattii* tenham sido mais eficientes que as outras espécies. No geral, *C. bacillisporus* e *C. deuterogattii* induziram níveis mais baixos de citocinas pró-inflamatórias TNF- α , IL-1 β e IL-6 e níveis mais altos de ERO do que aqueles induzidos pelas outras espécies. Curiosamente, Li e colaboradores [16] investigaram

a associação entre a variabilidade genética humana e a produção de citocinas em resposta a vários estímulos bacterianos, fúngicos e virais. Esses autores encontraram um polimorfismo de nucleotídeo único (SNP-rs4496335) relacionado aos níveis de TNF- α e com moderada associação aos níveis de IL-1 β e IL-6 em resposta ao *C. deuterogattii* (denominado *Cryptococcus* no artigo original), este SNP também foi relacionado com a expressão do gene IL1RN. Para confirmar estes resultados, os autores mostraram que a pré-incubação de PBMCs com o antagonista do receptor de IL-1 (IL-1Ra) inibe a indução de TNF- α por *C. deuterogattii* [16]. A capacidade de causar doença em humanos imunocompetentes e imunocomprometidos pode ser dependente tanto das características genéticas do patógeno quanto do hospedeiro [17-19]. Testou-se três isolados de cada espécie do complexo *C. gattii* e as diferenças observadas após a estimulação de PBMCs foram dependentes da espécie e citocina avaliada (dados não mostrados). Para *C. gattii s.s.* houve diferença significativa entre os isolados apenas na produção de IL-10 e para *C. bacillisporus* observou-se diferença significativa na produção de IL-1 β . A variabilidade genética do hospedeiro é um fator importante na determinação dos processos de doença, sendo assim, estudos adicionais focados na investigação do perfil genômico ou epigenético podem ajudar a esclarecer o quanto a variabilidade do hospedeiro e a variabilidade do patógeno contribui para o estabelecimento das doenças.

No **capítulo 5** observamos que todos os isolados de *C. neoformans s.l.* eram do sorotipo A e *mating-type* A, a maioria deles ($n = 172$; 78,5%) foram agrupados com a linhagem de referência AFLP1/VNI e 23 isolados (10,5%) compuseram um novo genótipo de AFLP denominado aqui como AFLP1C. O genótipo AFLP1A sub B foi agrupado em um cluster menor de microssatélites (MC) e 8 de 9 isolados deste cluster foram provenientes de pacientes HIV-positivos. Pan e colaboradores [20] observaram que a maioria dos isolados de um genótipo de microssatélites específico, denominado MC8, estava associada a pacientes HIV-positivos, e o MC17 apresentava a maioria dos isolados não-selvagens para flucitossina. A relação entre microssatélites e susceptibilidade antifúngica não foi observada no presente estudo. A análise de microssatélites revelou 129 genótipos diferentes em um conjunto de dados de 219 isolados, com 93 genótipos únicos. A maioria dos isolados ($n = 117$) foram agrupados em um MC principal, onde os isolados diferiram entre si em 1 de 9 marcadores de microssatélites analisados (ver Figura 2a, capítulo 5). O índice de diversidade de Simpson mostrou alta diversidade genética ($D = 0,9856$) entre os isolados de *C. neoformans s.s.*. Além disso, observamos que muitos genótipos de *C. neoformans s.s.* obtidos de diferentes regiões geográficas ao longo das três décadas de estudo, sugerindo que esses genótipos estão permanentemente estabelecidos no ambiente. Isolados clínicos e ambientais de *C. neoformans s.s.* estão

geneticamente relacionados, diferindo um do outro em um *locus* de microssatélite. A mesma relação foi observada para as populações de *C. neoformans s.s.* do Japão e da Tailândia [20]. Em contraste, um estudo epidemiológico realizado em Cuba revelou que isolados clínicos e ambientais de *C. neoformans s.s.* foram agrupados em diferentes MCs, mostrando diferentes nichos ambientais para este genótipo [21]. Uma limitação do presente estudo foi a presença de apenas 19 isolados ambientais. Experimentos futuros devem abordar um isolamento ambiental para que seja possível incluir mais isolados ambientais de *C. neoformans s.s.* na análise de microssatélites.

Os testes de suscetibilidade a antifúngicos *in vitro* mostrou que as espécies dos complexo *C. neoformans* e *C. gattii* variam em sua suscetibilidade, com valores de CIM mais elevados para o complexo de espécies *C. gattii* em comparação com o complexo de espécies *C. neoformans* [22-25]. Além disso, diferenças na susceptibilidade a antifúngicos foram encontradas também dentro dos complexos, com *C. deuterogattii* (AFLP6/VGII) e *C. neoformans s.s.* (AFLP1/VNI) apresentando menor suscetibilidade aos azólicos [26-28] quando comparados às outras espécies desses complexos. Os testes de suscetibilidade a antifúngicos *in vitro* realizados nos **capítulos 3 e 5** mostraram que todos os isolados de *C. deuterogattii* e *C. neoformans s.s.* são do tipo selvagem para todos os antifúngicos testados. No entanto, os isolados de *C. neoformans s.s.* apresentaram altas CIMs para fluconazol e flucitosina. A suscetibilidade *in vitro* pode não prever a resposta ao tratamento *in vivo*, portanto a terapia para criptococose é geralmente escolhida de acordo com a manifestação clínica e o estado imunológico do paciente, ao invés de considerar a espécie envolvida na infecção [29]. Entretanto, os testes de suscetibilidade são úteis para detectar casos especiais de resistência e, assim, auxiliar na adoção da melhor terapia antifúngica.

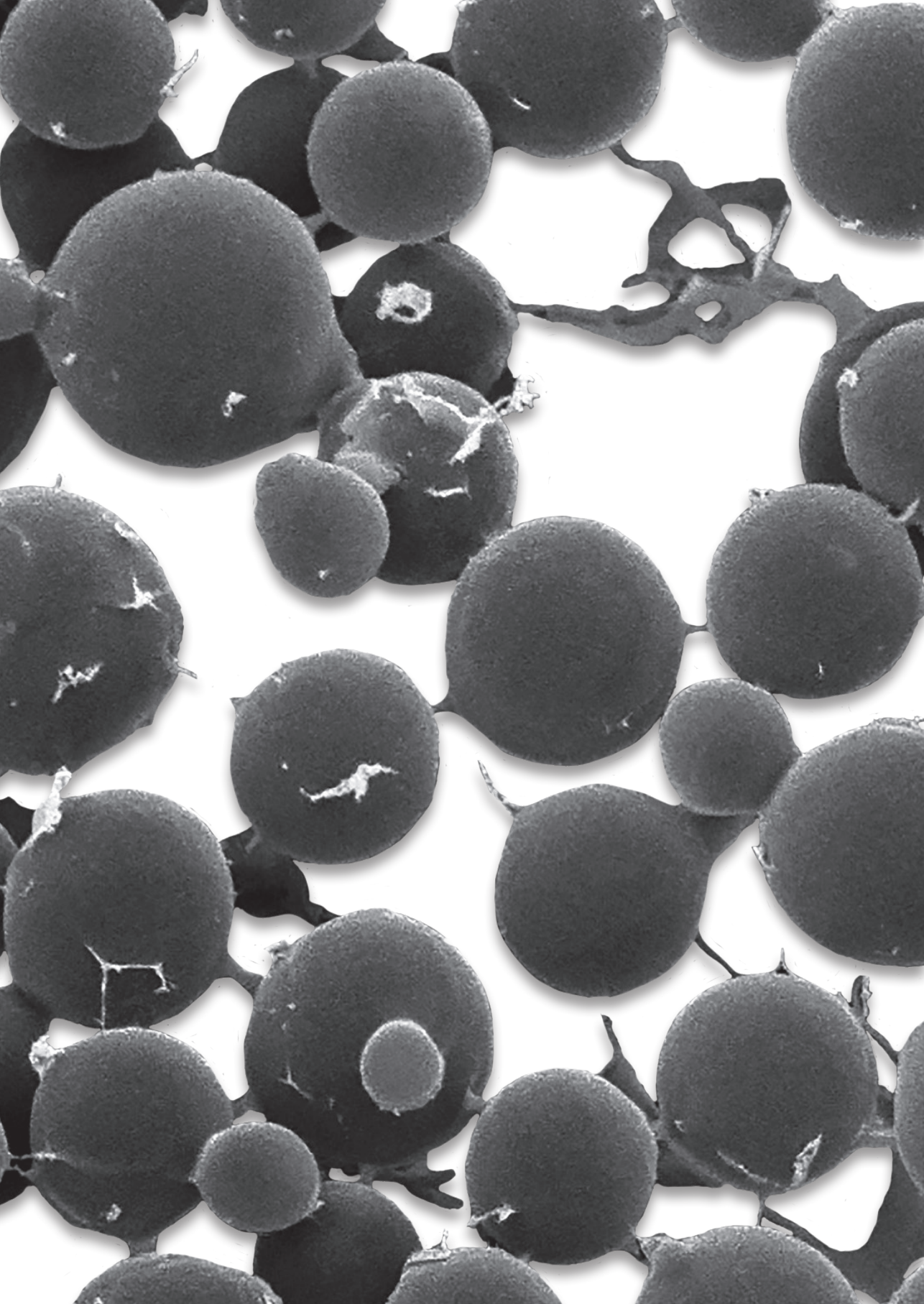
Como perspectivas futuras, a análise SCAR-MLST com os isolados de *C. deuterogattii* pode ser realizada para verificar a localização genômica dos marcadores dominantes dos novos padrões de AFLP, a fim de investigar se estes marcadores afetam aspectos biológicos do fungo, como a virulência. Por outro lado, ensaios de virulência podem ser realizados para investigar se UFU986 apresenta virulência comparável com a linhagem do surto da Ilha de Vancouver. Além disso, outros estudos devem se concentrar na investigação da presença de SNPs específicos relacionados com a suscetibilidade à criptococose, a fim de verificar como a variabilidade genética do hospedeiro e do patógeno contribuem para o estabelecimento da doença pelas diferentes espécies de *Cryptococcus*. Além disso, o isolamento ambiental de *C. neoformans s.s.* deve ser realizado com o objetivo de

incluir um número maior de isolados ambientais nas análises de microssatélites e, assim, obter informações mais robustas sobre a relação genética entre os isolados clínicos e ambientais de *C. neoformans* s.s. na região estudada.

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Chapter 7

Acknowledgments, Curriculum vitae and list of publications

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Curriculum vitae

Patricia F. Herkert was born on February 14th 1991 in Dionísio Cerqueira, a small city located in the country side of Santa Catarina state, Brazil. In this city, she grew up and started her studies. At age 17 she moved to the city Francisco Beltrão to study bachelor of biomedical sciences at “Universidade Paranaense – UNIPAR” between 2008-2011 and had the first contact with research in the “programa de iniciação científica - program of scientific initiation” offered by the university, when started to work with *Candida* spp. isolation from oral mucosa and antifungal susceptibility testing. During her graduation, she also participated in the program “monitoria acadêmica” to help other students to study human genetics, clinical biochemistry and clinical immunology.

In 2012 she moved to the city Curitiba to start her master’s degree in microbiology, in the Basic Pathology Department, at Federal University of Paraná, developing the project “Suscetibilidade *in vitro* e caracterização molecular de leveduras do gênero *Candida* isoladas de pacientes com candidemia” – Antifungal susceptibility and molecular characterization of *Candida* spp. from candidemic patients – with Prof. MD. PhD. Flávio Queiroz-Telles and Prof. PhD. Vânia Aparecida Vicente as supervisors. In March 2014 she defended her master’s thesis.

In June 2014, she started a fellowship in Microbiology, in the Basic Pathology Department, at Federal University of Paraná and began her studies in *Cryptococcus* spp. developing the project “Epidemiologia molecular de *Cryptococcus neoformans* s.l. e *C. gattii* s.l.” – Molecular epidemiology of *Cryptococcus neoformans* s.l. and *C. gattii* s.l. – with Prof. MD. PhD. Flávio Queiroz-Telles and Prof. PhD. Vânia Aparecida Vicente as supervisors. In 2015, she received a scholarship from Coordination for the Improvement of Higher Education Personnel (CAPES) (a foundation within the Ministry of Education in Brazil) to study abroad. Then, she moved to Nijmegen, the Netherlands, to work with MD. PhD. Jacques F. Meis and PhD. Ferry Hagen at CWZ, and with PhD. Leo Joosten at Radboudumc, where she studied between November 2015 and October 2016. From November 2016 until August 2017 she finalized her thesis work at the Federal University of Paraná and from September 2017 to January 2019 she worked as auxiliar professor at Faculdade de Pato Branco - FADEP, Brazil, teaching the subjects of cell biology, human genetics, biochemistry and microbiology for the Faculty of Medicine.

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