Genotypic diversity and clinical outcome of cryptococcosis in renal transplant recipients in Brazil

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ABSTRACT
Genotypic diversity and fluconazole susceptibility of 82 Cryptococcus neoformans and Cryptococcus gattii isolates from 60 renal transplant recipients in Brazil were characterized. Clinical characteristics of the patients and prognostic factors were analysed. Seventy-two (87.8%) isolates were C. neoformans and 10 (12.2%) were C. gattii. VN1 was the most common molecular type (40 cases; 66.7%), followed by VNII (9 cases; 15%), VGII (6 cases; 10%), VNB (4 cases; 6.7%) and VNI/II (1 case; 1.7%). The isolates showed a high genetic diversity in the haplotype network and six new sequence types were described, most of them for VNB. There was a bias towards skin involvement in the non-VNI population (P = 0.012). VGII isolates exhibited higher fluconazole minimum inhibitory concentrations compared to C. neoformans isolates (P = 0.008). The 30-day mortality rate was 38.3%, and it was significantly associated with fungemia and absence of headache. Patients infected with VGII had a high mortality rate at 90 days (66.7%). A variety of molecular types produce disease in renal transplant recipients in Brazil and highlighted by VGII and VNB. We report the clinical appearance and impact of the molecular type, fluconazole susceptibility of the isolates, and clinical characteristics on patient outcome in this population.

KEYWORDS Cryptococcus neoformans; Cryptococcus gattii; molecular type; renal transplantation; genotypic diversity

Introduction
Cryptococcosis is a life-threatening invasive fungal disease caused by the encapsulated yeasts, Cryptococcus neoformans and Cryptococcus gattii [1]. C. neoformans has a worldwide distribution affecting predominantly individuals with impaired cell-mediated immunity and C. gattii has a more limited environmental distribution and a higher percentage of disease within apparently normal hosts [1]. In renal transplant recipients, cryptococcosis is recognized as the second most common invasive fungal infection, with incidence rates ranging from 0.3% to 5.8% and overall mortality rates as high as 20–50% [2–11].

The nomenclature of C. neoformans/C. gattii species complexes is continuing to evolve under molecular evidence [12,13]. However, as a starting point, cryptococcosis is caused primarily by two species C. neoformans and C. gattii and currently these species can be further divided into ten molecular siblings known as VNI, VNII, VNB, VNIII, VNV, VGII, VGII, VGI, VGIII and VGIV [12] with a possible new molecular type designated VGV. The most widely utilized sequence-based genotyping method for the molecular identification of these complexes has been multilocus sequence typing (MLST). This method is robust and portable between laboratories [14,15].

Clinical comparative studies and understandings between different cryptococcal molecular types are still in their infancy and remain controversial whether or not these different molecular types represent specific characteristics in terms of clinical manifestations or attributable mortality rates [13,16–18]. Furthermore, most data related to strain distribution of C. neoformans and C. gattii species complexes in the transplant recipient relies on small series and case report [5,19–23].

The purpose of our study was to characterize the molecular types of C. neoformans and C. gattii isolated and to assess the clinical outcome of cryptococcosis and their molecular types in patients undergoing renal transplantation throughout Brazil. Interestingly, Brazil represents an environment with a diverse number of cryptococcal molecular types and likely has the most cryptococcal strain diversity of any country practicing routine kidney transplantation [24,25].
Results

Clinical characteristics

We enrolled a total of 60 renal transplant recipients followed for a median period of 4 months (0 days to 11 years). One patient had received a liver transplant allograft one year before kidney transplantation and another patient had undergone simultaneous pancreas-kidney transplantation. The clinical characteristics are outlined in Table 1.

Molecular characterization and clinical associations

We collected 82 isolates of C. neoformans/C. gattii species complexes from 60 renal transplant recipients. Ten

Table 1. Demographic and clinical characteristics of 60 renal transplant recipients infected by C. neoformans/C. gattii species complexes.

| Characteristics                                      | Value, % (no. of patients) |
|------------------------------------------------------|----------------------------|
| Age, average years (range)                           | 49 (21–71)                 |
| Male                                                 | 63.3 (38)                  |
| Ethnicity                                            |                            |
| White                                                | 60 (36)                    |
| Non-white                                            | 40 (24)                    |
| Retransplant*                                        | 6.7 (4)                    |
| Donor type                                           |                            |
| Deceased                                             | 60 (36)                    |
| Living                                               | 40 (24)                    |
| Immunosuppressive induction therapy                   | 40 (24)                    |
| Immunosuppressive agents received*                   |                            |
| Prednisone                                           | 100 (60)                   |
| Tacrolimus                                           | 56.7 (34)                  |
| Mycophenolic acid*                                   | 55 (33)                    |
| Azathioprine                                         | 26.7 (16)                  |
| Cyclosporine A                                       | 18.3 (11)                  |
| Rapamycin                                            | 8.3 (5)                    |
| Prior rejection                                      | 40 (24)                    |
| Diabetes mellitus                                    | 26.7 (16)                  |
| Active cytomegalovirus infection                     | 25 (15)                    |
| Hepatitis C infection                                | 18.3 (11)                  |
| Time to onset of infection after transplant, average months (range) | 30.5 (13 days to 17 years) |
| Sites of involvement                                 |                            |
| CNS                                                  | 83.3 (50)                  |
| Pulmonary                                            | 50 (30)                    |
| Skin, soft-tissue, or osteoarticular                  | 20 (12)                    |
| Fungemia                                             | 38.3 (23)                  |
| Disseminated infection                               | 63.3 (38)                  |
| Renal failure at baseline*                            | 56.7 (34)                  |
| Serum cryptococcal antigen titre, median (range)*    | 1:1024 (0–1:1024)          |
| Change in immunosuppression at diagnosis*            | 76.6 (44)                  |
| Antifungal therapy                                    |                            |
| Amphotericin B alone                                 | 60 (36)                    |
| Amphotericin B + SFC                                  | 18.1 (11)                  |
| Amphotericin B + fluconazole                         | 8.3 (5)                    |
| Fluconazole                                          | 3.3 (2)                    |
| None                                                 | 10 (6)                     |
| Mortality at 90 days                                 | 45 (27)                    |

Note: CNS, central nervous system; SFC, 5-flucytosine.

*Indicates prior receipt of a renal transplant.

**Indicates immunosuppressive agent that remained unchanged within 3 months of the onset of cryptococcosis.

†Includes mycophenolate mofetil or mycophenolate sodium.

‡Defined as the involvement of at least two noncontiguous organ systems or the presence of fungemia.

§Indicates creatinine ≥2 mg dl⁻¹ at the time of diagnosis of infection.

Data available for 35 patients.

Data available for 56 patients.

(12.2%) isolates were identified as C. gattii and 72 (87.8%) isolates as C. neoformans (Figure 1(A)). Forty-seven isolates were from cerebrospinal fluid (CSF), 23 from blood, 6 from pulmonary secretions, 5 from skin biopsy and 1 from urine. The distribution of different molecular types among the patients is depicted in Figure 1. The most common molecular type was VNI (51 isolates from 40 patients). For 20 episodes on which there were more than one isolate per patient, all but two exhibited similar molecular type within the same episode. In both patients infected by different molecular types we isolated VNI followed by VNII separated by 15 days in CSF or 44 days in blood. These two cases had their findings confirmed by three independent assays yielding the same results. In our entire cohort, only one isolate was diploid based on flow cytometry (VNI/VNII).

A total of 81 isolates were analysed by MLST and compared to MLST database. Nine allele types have been identified for the URA5 locus (1 new), 10 for LAC1 (1 new), 9 SOD1 (1 new), 14 for IGS1 region (3 new), 10 for CAP59 (1 new), 11 for PLB1 and 8 for GPD1 (1 new). Based on the combined analysis of the 7 MLST loci for C. neoformans, 20 sequence types (ST) were found, in which 6 were new (ST581, ST587, ST588, ST589, ST590 and ST591). Of those new ST, four were VNB molecular type and two VNII (Supplementary Table 1).

Figure 2 shows the phylogenetic tree of one representative sequence for each haplotype found for these transplant recipient isolates in relationship to reference strains. Our VGII isolates did not cluster with VGIIa, VGIIb or VGIIc.

For haplotype analyses we employed a dataset of 144 sequences (7 loci) from C. neoformans/C. gattii species complexes, being 81 generated in this study and 63 recovered from GenBank (Supplementary Table 1). Haplotype and nucleotide diversities were high in our overall dataset (number of haplotypes = 71; Hd = 0.961; π = 0.07003) and also specifically within our 81 transplant recipient isolates (Hd = 0.890; π = 0.03379). Remarkably, 24 out of 71 different haplotypes found in the full dataset for C. neoformans/C. gattii species complexes originated from transplant recipients. Only four haplotypes (H14, H15, H17 and H25) were identical to reference strains, revealing the high diversity of our isolates (Figure 3; Supplementary Table 1).

Regarding clinical correlations, patients infected by C. gattii were more likely to be a retransplant than those infected with C. neoformans (P = .046) (Table 2). Furthermore, patients infected with non-VNI molecular types were significantly more likely to have skin involvement than patients infected with VNI (40% vs. 10% patients, respectively; OR = 5.77; 95% CI = 1.47–22.56; P = .012). Fluconazole minimum inhibitory concentration (MIC) distributions for C. neoformans and C. gattii isolates varied as depicted in Table 3. Modal MICs were
patients infected with isolates exhibiting MICs ≥16 mg l⁻¹, with the higher mode for VGII (16 mg l⁻¹) and lowest mode for VNII (2 mg l⁻¹). For example, 13 out of 82 (15.85%) isolates exhibited fluconazole MIC values that were ≥16 mg l⁻¹, in which 8 (61.54%) were represented by molecular type VGII (Figure 3(A)). By multivariate analysis, fluconazole MIC values were higher in patients infected by C. gattii when compared to C. neoformans (OR = 3.79; 95% CI = 2.07–7.00; p < .001, log rank test) (Figure 4).

Discussion

Our retrospective analysis describes 60 cases of cryptococcosis among renal transplant recipients documented during a period of 26 years. The patients presented with high rates of fungemia (38.3%), common disseminated infection (63.3%), and substantial 90-day mortality (45%). These data are comparable to some prior studies evaluating cryptococcosis in renal transplant recipients [3,5,7,8,26,27]. However, lower rates of mortality have been recently reported among all types of solid transplant recipients in high resource-available countries [2,9,11,26–28]. Furthermore, in our cohort of transplant recipients infected by C. gattii, there was an impressive 66.7% mortality rate within 90 days. Similarly, other investigators have found high rates of dissemination (63.6%) and a high 90-day mortality (36%) for solid organ transplant (SOT) recipients infected by C. gattii [19]. These observations emphasize the potential need for clinicians to know the identification of the species of Cryptococcus in an individual transplant recipient infection for prognostic determination and possible management alterations.

We observed clinically that fungemia and absence of headache were significantly correlated with decreased survival in our kidney transplant recipients. These characteristics are likely to identify a patient with a high burden of yeasts and/or late diagnosis. These clinical characteristics have been found to be poor prognostic features in other patient populations [29,30].

In Brazil, most cryptococcal molecular types in infections are represented. Indeed, MLST and whole-genome based population analyses suggest that Brazil could be a global centre for diversity of C. neoformans/C. gattii species complexes and even a location for species origin [21,25,31–33]. However, studies addressing the distribution of species molecular types of Cryptococcus infecting transplant recipients remain scarce worldwide. In the setting of SOT, one study in China revealed 9 cases of VNII and a recent study described 10 strains of molecular type VGII and one of VGI from the Pacific Northwest of USA outbreak. In this last study, 18% of the strains in transplant recipients were C. gattii [5,19]. We found in our cohort of renal transplant recipients that C. gattii represented 12% of isolates (VGII). While VNI was the most common molecular type infecting our patients (66.7%), some genotypic diversity with C. neoformans was also found as we detected 14 VNII and 6 VNB isolates. The molecular predominance of VGII may...
represent either a specific tropism for transplant recipients and/or increased resistance to tacrolimus/cyclosporine or more likely higher exposure to VGII in the environment over the other VG molecular types. A higher frequency of *C. gattii* disease observed in retransplantation recipients supports the hypothesis that cryptococcal disease of immunocompromised hosts with *C. gattii* may be more a primary disease while *C. neoformans* disease more frequently represents secondary or reactivation disease [4]. Surprisingly we found VNB isolates of *C. neoformans* in this Brazilian cohort, a molecular type that was considered to be geographically restricted to Africa [34]. However, recently, almost a dozen clinical and environmental isolates from Italy, Portugal, China and Brazil have been identified as VNB strains [20,35–39].
fact, a recent study comparing Brazilian VNB isolates from transplant recipients with African isolates showed a high diversity within these isolates, except for one isolate from Brazil that nested deeply within the African clade on the phylogeny tree [31]. This isolate was recovered from a mulatto patient living in São Paulo and might have corresponded to a recent migration event. However, VNB isolates as a whole may support the fact that their geographical niche was separated during the Pangea period in which continents split [40].

The Next Generation MLST (NGMLST) methodology and primers that we used were not able to properly sequence the \textit{GPD1} locus for VGII molecular type as previously reported [14]. Another issue for our genotyping analysis was our inability to precisely separate our VGII isolates into three distinct clonal lineages (VGIIa, VGIIb and VGIIc). MLST and whole-population genome studies comparing the VGII outbreak strains with VGII isolates from other regions showed that isolates, especially from south of Brazil, have not clustered with any of the three specific lineages [25,33,41].

The clinical impact of different cryptococcal molecular types on patient outcome is still unknown. Controversial results have been generated in the correlation between molecular types and virulence based on experimental studies [13,16,18] and clinical data [17,20,42,43]. We failed to demonstrate substantial differences in clinical presentation or outcome by molecular types, except that non-VNI molecular strains were significantly more likely to show skin involvement than patients infected by VNI strains. There were also no significant differences in outcomes between the \textit{C. neoformans} and \textit{C. gattii} infections with the important caveat that there were fewer \textit{C. gattii} strains and all were VGII. Consequently, the statistical power to detect differences in our study is limited. However, these initial observations support the hypothesis that virulence is not consistently associated with a single major molecular type.

Figure 3. Median-joining haplotype network of 144 isolates of \textit{C. neoformans}/\textit{C. gattii} species complexes (81 isolates originated in this study in addition to 63 reference strains recovered from literature), covering all the concatenated loci \textit{CAP59}, \textit{LAC1}, \textit{PLB1}, \textit{SOD1}, \textit{URA5}, \textit{TEF1} and \textit{IGS1} sequences. The isolates are coded, and their frequencies are represented by (A) fluconazole MIC $\geq$ than 16 mg l$^{-1}$ from transplant recipients isolates or (B) 90-days mortality of transplant recipients. The size of the circumference is proportional to the haplotype frequency. The black dots (median vectors) represent unsampled or extinct haplotypes in the population. Further information about isolate source and GenBank accession number can be found in the Supplementary Table 1.
Table 2. Comparisons of infections due to C. neoforms and C. gattii complex in 60 renal transplant recipients.

| Characteristics (N°. patients) | C. neoforms infection % (N°. of patients) n = 54 | C. gatti infection % (N°. of patients) n = 6 | P-value | OR 95% CI | P-value |
|-------------------------------|-----------------------------------------------|-----------------------------------------------|---------|---------|---------|
| Age, average years (range)    | 49 (21–69)                                    | 50.5 (32–71)                                  | 0.796   |         |         |
| Male                          | 66.7 (36)                                     | 33.3 (2)                                     | 0.179   |         |         |
| White                         | 61.1 (33)                                     | 33.3 (2)                                     | 0.223   |         |         |
| Living in the capital before infection | 27.8 (15)                                | 6.3 (1)                                      | 1.0     |         |         |
| Northeast of Brazil as place of birth | 31.5 (17)                                | 50 (3)                                       | 0.390   |         |         |
| Retransplant                  | 3.7 (2)                                       | 33.3 (2)                                     | 0.046   |         |         |
| Cytomegalovirus infection     | 38.9 (21)                                     | 50 (3)                                       | 0.675   |         |         |
| Time to onset of infection after transplant, average months (range) | 30 (13 days–7 years) | 37.5 (188 days–7 years) | 0.667 |
| Sites of involvement          |                                               |                                              |         |         |         |
| CNS (56)                      | 90.6 (48)                                     | 66.7 (2)                                     | 0.293   |         |         |
| Pulmonary (59)                | 48.1 (26)                                     | 80 (4)                                       | 0.353   |         |         |
| Skin, soft-tissue, or osteoarticular | 16.7 (9)                                 | 50 (3)                                       | 0.088   |         |         |
| Encephalitis                  | 35.2 (19)                                     | 66.7 (4)                                     | 0.191   |         |         |
| Disseminated infectiona       | 61.1 (33)                                     | 83.3 (5)                                     | 0.4     |         |         |
| Renal failure at baselineb    | 53.7 (29)                                     | 83.3 (5)                                     | 0.221   |         |         |
| CNS image abnormality         | 20 (7)                                        | 0 (0)                                        | 1.0     |         |         |
| Serum cryptococcal antigen titre ≥ 1:512 | 57.1 (16)                                 | 100 (3)                                      | 0.265   |         |         |
| Mean duration of hospitalization, ±SD (range), days | 36.8 ± 38.19 (1–245) | 9.33 ± 11.89 (8–33) | 0.087 |
| Duration of antifungal induction therapy, average days (range) (54) | 26.3 (1–219) | 16 (4–28) | 0.504 |
| Total duration of antifungal therapy, average days (range) (51) | 115 (1–635) | 288.5 (193–384) | 0.453 |
| Fluconazole MIC ≥ 16 mg l−1 | 7.4 (4)                                       | 66.7 (4)                                     | 0.002   | 12.62   | 1.97–80.99 | 0.008 |
| Mortality at 90 days          | 42.6 (23)                                     | 66.7 (4)                                     | 0.394   |         |         |

Note: CNS, central nervous system; SD, standard deviation; MIC, minimum inhibitory concentration.
aIndicates prior receipt of a renal transplant.
bDefined as the involvement of at least two noncontiguous organ systems or the presence of fungemia.
cIndicates creatinine ≥ 2 mg dl−1 at the time of diagnosis of infection.

or subtype but may be more related to the distinct properties of individual isolates.

Fluconazole susceptibility profile is a potentially important clinical issue but its relevance in terms of precisely determining prognosis is still controversial, especially in the setting of the transplant population [5,19,44,45]. In general, direct correlation between species type and antifungal susceptibility has varied [18,21,46–48]. Our VGII isolates showed high fluconazole MICs. Furthermore, in our study, infection by C. neoforms/C. gattii species complex isolates exhibiting high fluconazole MICs did correlate with a worse patient survival rate. However, the precise clinical correlation of in vitro antifungal susceptibility testing of Cryptococcus and break points remains to be further defined [49–53].

In conclusion, cryptococcosis in the setting of SOT in less-resourced countries should be considered a life-threatening fungal disease with substantial mortality rates. The high mortality is probably due to late diagnosis, suboptimal antifungal therapy, including lack of 5FC, as well as the virulence properties of these fungi in SOT. In fact, this transplant population in Brazil represents a relatively uniform host with high rates of mortality for cryptococcosis that may allow for the detection and characterization of particularly virulent strains. With this diversity of strains and a similar high-risk host population for mortality, further examination of these transplant cohorts in Brazil and their fungal strains may give us signals into how these yeasts specifically cause aggressive disease.

Table 3. Fluconazole MIC distribution for 82 isolates of C. neoforms/C. gattii species complexes tested.

| Molecular type or specie | Number of isolates | Mean MIC mg l−1 | Interval MIC mg l−1 | MIC50 mg l−1 | MIC90 mg l−1 | No. of isolates for which the MIC (mg l−1) was: |
|-------------------------|--------------------|-----------------|--------------------|-------------|-------------|---------------------------------|
|                         |                    |                 |                    |             |             | ≤0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | ≥64 |
| All isolates            | 82                 | 8.70            | 0.25–64            | 8            | 16          | 1     | 3   | 11  | 20 | 34 | 9  | 2  | 2  |     |     |
| C. neoforms             | 72                 | 6.18            | 0.25–16            | 8            | 8           | 1     | 3   | 10  | 19 | 33 | 5  |     |     |     |
| VNI                    | 51                 | 7.22            | 0.25–16            | 8            | 8           | 1     | 3   | 32  | 15 | 28 | 5  |     |     |     |
| VNB                    | 14                 | 2.50            | 1–8                | 2            | 4           | 3     | 8   | 2   | 1  |     |     |     |     |
| VNI/II                 | 6                  | 6.86            | 4–8                | 8            | 8           | 2     | 4   |     |     |     |     |     |     |
| C. gattii – VGII        | 1                  | 1               |                    | 1            |             | 1     |     |     |     |     |     |     |     |

Note: MIC, minimum inhibitory concentration.
aThe modal MIC for each distribution is underlined.
Materials and methods

Isolates

We evaluated all *C. neoformans* and *C. gattii* strains isolated from patients who underwent renal transplantation and subsequently developed cryptococcosis from 1987 to 2013 at São Paulo Hospital or the Kidney Hospital in São Paulo. The isolates were stored in cryopreservative medium at $-70^\circ$C with Yeast Peptone Dextrose (YPD) and 20% glycerol at the Special Mycology Laboratory – Federal University of São Paulo.

Clinical data and definitions

The following variables were collected from the medical records using a standard clinical report form: demographic data, complications prior to and after transplantation, immunosuppressive regimens, antifungal therapy, and laboratory data. Disseminated cryptococcosis was defined as the involvement of at least two noncontiguous organ systems or the presence of fungemia [6]. Primary therapy was defined as the first systemic antifungal regimen administered for at least two consecutive days [54]. The study was approved by the local ethic committee (UNIFESP Number: 318847, 2013).

Genotyping

A single colony of each isolate was isolated, grown in YPD broth and frozen at $-70^\circ$C with glycerol. To isolate genomic DNA, the isolates were streaked from these frozen stocks onto fresh YPD agar, grown for 2–4 days. Next, several colonies of each sample were

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Table 4. Variables associated with 30-day mortality after cryptococcosis in 60 renal transplant recipients.

| Variables (N°. patients) | Survival, % (n = 37) | Death, % (n = 23) | P-value | OR (95% CI) | P-value |
|--------------------------|----------------------|------------------|---------|-------------|---------|
| Age, mean, ±SD, years (60) | 48.1 ± 12.51 | 49.6 ± 12.94 | 0.657 | | |
| Male (38) | 56.8 | 73.9 | 0.180 | | |
| Induction immunosuppressive therapy (24) | 27 | 60.9 | | 0.009 | |
| Deceased donor (36) | 48.6 | 78.2 | | 0.023 | |
| Prior rejection (24) | 43.2 | 34.8 | | 0.515 | |
| Cytomegalovirus infection* (7) | 13.5 | 8.7 | | 0.681 | |
| Receipt of a calcineurin-inhibitor agent* (45) | 64.9 | 91.3 | | 0.021 | |
| Receipt of a tacrolimus* (34) | 45.9 | 73.9 | | 0.034 | |
| Duration of symptoms before diagnosis, mean, ±SD (56) | 35.4 ± 42.69 | 29.2 ± 78.74 | | 0.705 | |
| Time to diagnose after admission, mean, ±SD (57) | 4.7 ± 9.09 | 3.6 ± 4.65 | | 0.585 | |

Sites of involvement

| CNS (50) | 89.2 | 89.5 | 1 |
| Pulmonary (30) | 40.5 | 68.2 | 0.04 |
| Skin or soft-tissue (12) | 24.3 | 13 | 0.34 |
| Disseminated infection (38) | 54.1 | 78.3 | 0.059 |

Fungemia (23)

| 18.9 | 69.6 | <0.001 | 3.79 | 1.03–13.91 | 0.044 |

C. gattii infection (6)

| 5.4 | 17.4 | 0.191 |

Non-VNI genotype (20)

| 32.4 | 34.5 | 0.851 |

Creatinine at admission $\geq$2 mg dL$^{-1}$ (34)

| 51.4 | 65.2 | 0.292 |

Somnolence at admission* (19)

| 27.3 | 58.8 | 0.029 |

Confusion at admission* (19)

| 30.3 | 52.9 | 0.318 |

Headache at admission* (30)

| 72.7 | 35.3 | 0.01 | 0.13 | 0.04–0.42 | 0.001 |

Intracranial hypertension (16)

| 48.1 | 33.3 | 0.7 |

Respiratory failure* (7)

| 6.7 | 40 | 0.08 |

CSF cell count*; mean, ±SD, mm$^3$ (42)

| 138.5 ± 250.04 | 101.1 ± 92.8 | 0.664 |

CSF glucose ratio*; mean, ±SD, mg dL$^{-1}$ (41)

| 47.3 ± 24.63 | 39.2 ± 31.89 | 0.420 |

Positive CSF India ink* (23)

| 42.4 | 64.3 | 0.170 |

CSF antigen titre $>1.512^+$ (24)

| 43.3 | 84.6 | 0.012 |

Positive pulmonary culture* (9)

| 33.3 | 100 | 0.021 |

AMBd as primary therapy (33)

| 97.3 | 100 | 1 |

Combination therapy* (16)

| 24.3 | 41.2 | 0.208 |

Change in immunosuppressive regimen after infection (44)

| 80.6 | 75 | 0.737 |

Discontinuation of tacrolimus after infection* (24)

| 41.4 | 80 | 0.025 |

Graft loss within 30 days (7)

| 13.5 | 100 | 0.028 |

Fluconazole MIC $\geq$16 mg L$^{-1}$ (8)

| 25 | 75 | 0.045 |

Note: OR, odds ratio; CI, confidence interval; SD, standard deviation; CNS, central nervous system; CSF, cerebrospinal fluid; AMBd, amphotericin B deoxycholate; MIC, minimum inhibitory concentration.

*Infection occurring within 6 months of the onset of cryptococcosis.

$^+$At cryptococcosis diagnosis.

*Data was available for 56 patients.

$^+$Data was available for 59 patients.

*Data was available for 50 patients.

*Data was available for 36 patients.

*Data was available for 30 patients.

$^+$Data was available for 47 patients.

*Data was available for 43 patients.

*Data was available for 13 patients.

*Data was available for 54 patients, at least 2 consecutive days of the same antifungal therapy.

*Data was available for 44 patients.

*Data was available for 39 patients.

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used to extract and purify the genomic DNA, using the MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). We analysed the sequences from eight loci, including seven standard gene regions (CAP59, LAC1, PLB1, SOD1, URA5, TEF1, GPD1) and the intergenic spacer region 1 (IGS1) of the nuclear ribosomal RNA gene. The primer sequences and the preparation of the sequencing libraries were based on the NGMLST method previously described and the raw sequencing data was processed using MLSTEZ [14]. The allele type of each generated consensus sequence for every locus was determined using the MLST database (http://mlst.mycologylab.org).

The phylogenetic analysis was performed using MEGA 7.0 [55]. The evolutionary relationships, with 1000 bootstrap replicates of the concatenated nucleotide sequences, were inferred using maximum likelihood and the neighbor-joining methods [56]. Major molecular types were confirmed according to phylogenetic clustering with reference strains. Evolutionary relationships at the intraspecific level were evaluated using haplotype networks in order to visualize differences and diversity among isolates. Haplotype and nucleotide diversities were estimated using DNAsp v5.0 [57]. Median-joining networks for the dataset were obtained and visualized using the software Network 5.0 (Fluxus Technology). Flow cytometry was used to check ploidy of the hybrid isolate [58].

Antifungal susceptibility testing

The determination of MICs for fluconazole was performed using the CLSI broth microdilution assay, according to the M27-A3 document [59]. Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258 were included as quality controls. Assays were performed in RPMI with endpoints read after 72 h at 35°C. The MIC was defined as the lowest concentration that produced 50% growth inhibition compared with the drug-free growth control. The interpretation of MIC values was based on epidemiological cutoff values [60].

Statistical analysis

Continuous data were presented as either the mean ± SD or the median and range, and categorical data were presented as proportions. Univariate analyses were performed to compare categorical variables using either the Chi-square or Fisher’s exact test as appropriate and continuous variables by Student’s t-test. Variables whose univariate test result had a P-value <.1 were considered candidates for the multivariate model. Binary logistic models were generated using forward stepwise selection for factors associated with death and comparisons between molecular types and species. All analyses were performed using SPSS software for Windows, version 22 (SPSS, Chicago, IL). A value of P ≤ .05 was considered statistically significant.

Data availability

The sequences of all newly identified allele types have been submitted to the MLST database (http://mlst.mycologylab.org) and GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

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