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Characterization of Clinical and Environmental Isolates of *Cryptococcus neoformans/Cryptococcus gattii* Complex Maintained in Yeast Culture Collection in São Paulo, Brazil

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Abstract

Objective: As isolates of *Cryptococcus* are frequently kept collection stocks in institutions, sometimes without proper characterization, we sought to determine the genotype profiles, protease and phospholipase activities "*in vitro*" and the susceptibility testing for azoles and amphotericin B. **Methodology:** 84 isolates from several regions of Brazil (40 samples from clinical origin and 44 isolates from environmental origin) were maintained at the microorganism's bank of the Biomedical Science Institute (ICB-USP) of the São Paulo University, in São Paulo, Brazil. This isolates was submitted fungal strains determination, DNA extraction and purification, determination of genotype by *URA*5 gene RFLP of CGB-positive isolates, protease and phospholipase activity and susceptibility to antifungals. **Results:** Of six CGB positive isolates tested by RFLP-PCR, only four presented a genomic profile consistent *C. gattii* species

(VGII), while two other were *C. neoformans* (VNI and VNIII), indicating the existence of canavanine-resistante *C. neoformans* isolates in the culture collections. The clinical isolates secreted higher levels of phospholipase and environmental isolates but no differences were observed for the protease levels. Almost all isolates were sensible to azoles and amphotericin B. **Conclusion:** We point out in this research the existence of *C. neoformans* strains resistant to canavanine and intrinsic characteristic of *C. gatti.* These results demonstrate the importance to perform a detailed characterization of isolates kept in culture collections.

Keywords

Cryptococcus Complex, Genotyping, Protease, Phospholipase, Etest, Culture Collection

1. Introduction

Basidiomycetous yeasts of the genus *Cryptococcus* comprise seventy species, some of which are associated with invasive infections in humans and animals, such as *Cryptococcus neoformans* and *Cryptococcus gattii* which belong to the *Cryptococcus neoformans*/*Cryptococcus gattii* complex [1] [2] [3]. *C. neoformans* comprises two varieties named variety *grubii* (serotype A) and variety *neoformans* (serotype D), while *C. gattii* has two serotypes (B and C). They have distinct, though occasionally overlapping, ecological niches, with *C. neoformans* often been isolated from pigeon (*Columba livia*) nests and droppings, and *C. gattii* often been isolated from decaying trees and soil [4] [5] [6] [7].

C. neoformans and *C. gattii* can be phenotypically differentiated by L-canavanine glycine bromothymol blue (CGB) agar medium [8] [9] while its genotype-based differentiation can be performed by PCR fingerprinting [10], restriction fragment length polymorphism (RFLP-PCR) typing [11] [12], intergenic spacer sequencing [13], amplified fragment length polymorphism analysis (AFLP) [14] and multilocus sequence typing (MLST) [15] [16].

Phenotypic characteristics that are associated with the virulence of *Cryptococcus* have also been studied since they play important roles in the fungal pathogenesis [17]. These include the capsule thickness [18] [19] [20], melanin production [21], urease, protease and phospholipase activities [22] [23] [24] and susceptibility profile to different antifungals [12] [25] [26] [27].

Considering that the biological characteristics of many *C. neoformans* complex stocks in Brazil are not generally tested in detail, and that some isolates are recorded with outdated taxonomic nomenclatures or even misidentified, they need to be constantly reevaluated.

2. Objective

We sought to determine the genotype profiles, *in vitro* protease and phospholi-

pase activities and the susceptibility testing to azoles and amphotericin B, in clinical and environmental isolates of the *C. neoformans*/*C. gattii* complex, which are kept stored at the microorganism's bank of the *Instituto de Ciências Biomédicas* (ICB-USP) of the *Universidade de São Paulo* (USP), in São Paulo, Brazil.

3. Material and Methods

3.1. Fungal Strains

This study was done on 84 isolates from several regions of Brazil. Of them, 40 samples were isolated from either cerebrospinal fluid or blood samples of human and animal cryptococcosis cases and 44 samples were isolated from environment bird droppings or the leaves of *Eucalyptus*. They were formerly identified as *C. neoformans* and were maintained in chloramphenicol-supplemented Sabouraud dextrose agar slants plus mineral oil, at the microorganism's bank at the Laboratory of Pathogenic Yeasts of the ICB-USP. Standard strains ICB 110—*C. neoformans* var. *grubii*, ICB134—*C. neoformans* var. *neoformans*, ICB 162—*C. gattii* and ICB 12A—*Candida albicans* were used in all tests. The isolates were identified by their urease activity, growth in L-canavanine glycine bromothymol blue (CGB) medium and assimilation of nitrate [3]. The institutional review board of ICB-USP committee approved this study (Protocol 367/2010).

3.2. DNA Extraction and Purification

DNA genotyping was done on CGB positive isolates. The DNA extraction was done according Martins *et al.* (2007) [28]. Briefly, the isolates were dissolved in a lysis buffer containing 10 mM Tris-HCl pH 8.0, 10 mM ethilenediaminetetraacetic acid (EDTA) pH 8.0, 0.5% sodium dodecyl sulfate (SDS), 0.01% N-laurilsarcozyl and 100 μg/mL proteinase K.

The mixtures were briefly vortexed and incubated at 56˚C for 2 hours or until the cell lysis was completed. DNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1) method and precipitated with isopropanol [29]. The DNA pellet was washed with 70% ethanol, centrifuged for 10 min at 10,000 g and air-dried. The DNA was suspended in 200 μL of sterile ultra-pure water containing RNase 20 μg/mL, kept at 4˚C overnight and stored at −20˚C. DNA concentrations were determined in O.D. at 260 nm, while DNA purity was determined by the ratio of O.D. at 260 and 280 nm. For PCR amplification one μL of each DNA sample (85 - 90 ng) was used as DNA template [29] [30].

3.3. Determination of Genotype by *URA*5 Gene RFLP of CGB-Positive Isolates

Amplification of the *URA*5 gene was carried out with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), according manufacturer's instructions in a final volume of 25.0 μL. Each reaction mixtures consisted of 12.5 μL of GoTaq® Green Master Mix, 0.7 μL of each primer: *URA*5

(5′-ATG/TCC/TCC/CAA/GCC/CTC/GAC/TCC/G-3′) and SJ01 (5′-TTA/AGA/CCT/CTG/AAC/ACC/GTA/CTC-3′) (Invitrogen, Carlsbad, CA, USA), 1.0 μL of DNA and 10.1 μL of nuclease-free water. The amplification of the fragments was performed in a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems TM, Thermo Fisher Scientific, Waltham, MA, USA). The cycling condition was 95˚C for 4 min initial denaturation, followed by 34 cycles of 94˚C for 45 sec of denaturation, 57˚C for 1 min of annealing, 72˚C for 1 min of extension, followed by a final cycle of 72˚C for 10 minutes of final extension. Subsequently, amplification products were mixed with one fifth volume of loading buffer (15% ficoll 400, 0.25% orange G, MiliQ water; 2.0 μL), 15.0 μL of amplified PCR products double digested using *Cfr*13I (isoschizomer of *Sau*96I) (10 U μL; 1.0 μL) and *Hha*I (20 U μL, 1.0 μL), and 1.0 μL of sterile MiliQ water in a final volume of 20.0 μL (Thermo Fis her Scientific, Waltham, MA, USA), and then incubated in dry at 37˚C for 3 h or overnight. The restriction fragments were then separated by 2% agarose gel electrophoresis at 100 V for 40 min. RFLP patterns were assigned visually by comparison with the patterns obtained from the standard reference isolates from eight molecular types: VNI-VNIV for *C. neoformans*, and VGI-VGIV for *C gattii* [30].

3.4. Protease and Phospholipase Activity

Protease activity was performed in triplicate according to Rüchel *et al.* (1982) [31]. Briefly, a culture medium dish containing sterile bovine albumin fraction V (Sigma-Aldrich, St. Louis, MO, USA) was distributed into Petri dishes. Each isolate was inoculated on the central surface of the plate and they were incubated at 32˚C in 5% CO_2 for 15 days and examined every two days until the 15th day. A translucent halo formed around the yeast colony indicated protease activity. Proteolytic activity (Pz value) was calculated as the ratio of the diameter of the colony (Dc) and to the diameter of the colony plus that of the degradation zone diameter (Dzd) (Pz = Dc/Dc + Dzd). Therefore, Pz = 1.00 indicated no activity (negative); Pz < 1.00 and > 0.64 indicated median activity (positive) and Pz < 0.64 indicated a strong activity (strongly positive) for protease activity.

Phospholipase activity was performed in triplicate by the egg yolk agar test [32]. Briefly, the isolates were previously cultured during 48 hours Sabouraud Dextrose Agar Supplemented with chloramphenicol (Difco Laboratories Inc., Detroit, MI, USA) and then inoculated on the central surface of the phospholipase test plate and were incubated at 32˚C in 5% CO_2 for 15 days. An opaque halo of precipitation around the colony was indicative of phospholipase activity. The plates were examined for phospholipase activity every two days up to the 15th day. Phospholipase activity (Pz value) was calculated as the ratio of the diameter of the colony (Dc) and to the diameter of the colony plus that of the precipitation zone diameter (Pzd) (Pz = Dc/Dc + Pzd). Therefore, Pz = 1.00 indicated no activity (negative) strain; Pz < 1.00 and > 0.64 indicated median activity (positive) and Pz < 0.64 indicated a strong activity (strongly positive) for phospholipase activity.

3.5. Susceptibility to Antifungals

Etest strips for fluconazole, itraconazole, voriconazole, posaconazole and amphotericin B were provided by AB Biodisk (Solna, Sweden) and the assay was done according the manufacturer's instructions (http://www.abbiodisk.com/). Sterile fungal cell suspensions were adjusted to the turbidity of a 0.5 McFarland standard. Sterile and non-toxic swabs were dipped in these cell suspensions and inoculated on the surface of Petri dishes containing RPMI 1640 (Gibco-BRL, Grand Island, NY, USA) supplemented with 1.5% agar and 2% glucose (J.T.Baker® Chemicals, Center Valley, PA, USA), buffered with 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, MO, USA) [33] [34].

Etest strips were applied to each Petri dish, and the plates were incubated at 35˚C in 5% CO_2. The strip MICs were read at 48 and 72 hours of incubation. The azole minimum inhibitory concentration (MIC) was read at the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip. Any growth, such as microcolonies, throughout a discernible inhibition ellipse was ignored [34]. The amphotericin B MICs were read at the point at which the zone of almost complete inhibition intersected the strip. All susceptibility tests were repeated twice.

The interpretative criteria used for susceptibility to fluconazole, itraconazole, voriconazole, posaconazole and amphotericin B antifungals was done according to Clinical and Laboratory Standards Institute (CLSI) [35]: for fluconazole, susceptible (S) ≤ 8 μg/mL; susceptible dose dependent (S-DD) 16 - 32 μg/mL; resistant (R) ≥ 64 μg/mL; for itraconazole, S ≤ 0.125 μg/mL; S-DD 0.25 - 0.5 μg/mL; R ≥ 1 μg/mL; for voriconazole S ≤ 1 μg/mL; S-DD 2 μg/mL; R ≥ 4 μg/mL; for posaconazole S ≤ 1 μg/mL; S-DD 2 μg/mL; R ≥ 4 μg/mL. Since no breakpoints have been published for amphotericin B, breakpoints chosen for it were (S ≤ 1 μg/ml, S; intermediate (I) 2 μg/mL; R ≥ 4 μg/mL) [36].

4. Results

The isolates of clinical and environmental origin were re-identified and were consistent with the colonial and cellular morphology and physiological characteristics of *C. neoformans* complex, as proposed by Kurtzman *et al.* (2011) [3]. The isolates were also cultured on CGB medium, for differentiation of *C. neoformans* and *C. gattii*. Of the 40 isolates of clinical origin, 36 (90.0%) were identified as *C. neoformans* and four (10.0%) as *C. gattii.* On the other hand, of the 44 isolates of environment origin, 42 (95.5%) were identified as *C. neoformans* and two isolates (4.5%) as *C. gattii.*

4.1. RFLP of *URA*5 Gene of CGB-Positive Isolates

Of the six isolates formerly identified as *C. gattii* four were classified as genotype VGII (serotype B and C) according to **Figure 1**, but two were identified as *C. neoformans,* one as genotype VNI (serotype A, var. *grubii*) and one as genotype VNIII (hybrid serotype AD) (**Figure 2**).

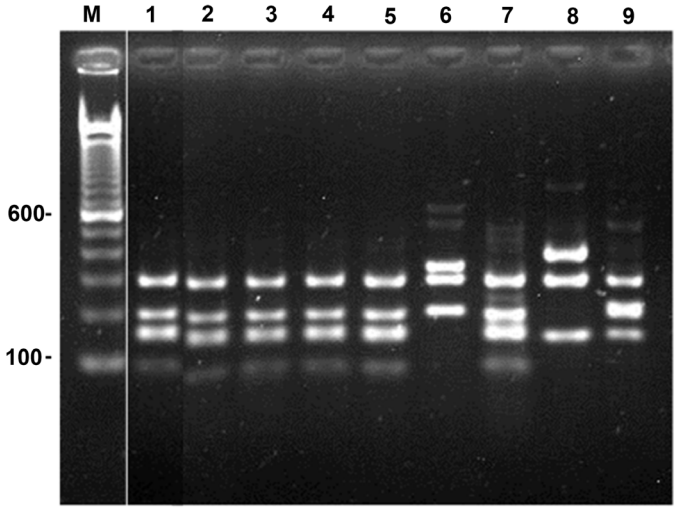

Figure 1. RFLP profiles of the URA5 genes from *Cryptococcus gattii* obtained by double-digestion with *Hha*I and *Cfr*13I. Lanes 1 - 5: isolates 161, 182, 183, 184 e 189 respectively. Lanes 6 - 9 standards genotypes VGI, VGII, VGIII and VGIV respectively. M-100bp DNA ladder (Invitrogen).

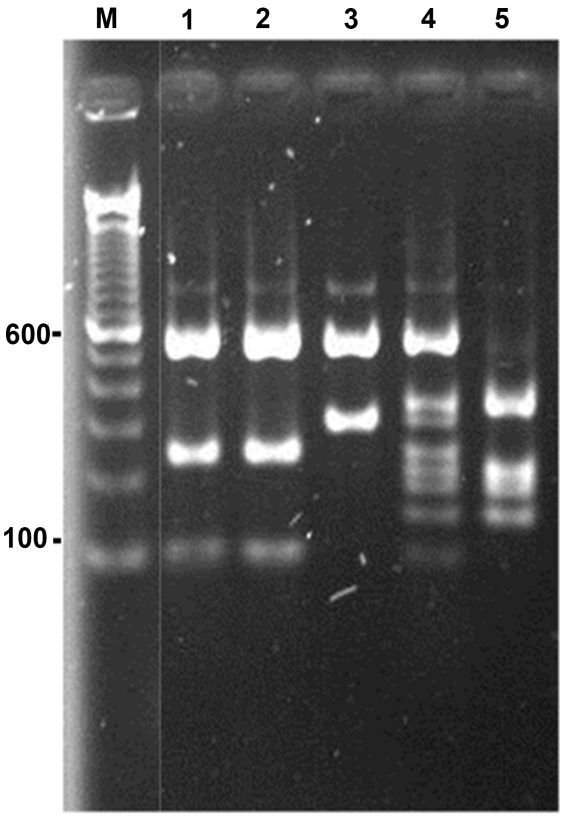

Figure 2. RFLP profiles of the URA5 genes from *Cryptococcus neoformans* obtained by double-digestion with *Hha*I and *Cfr*13I. Lane 1 isolate 164 Lanes 2 - 5 standards genotypes VNI, VNII, VNIII and VNIV respectively. M-100bp DNA ladder (Invitrogen).

4.2. Protease and Phospholipase Activity

As shown in Table 1, of the 40 isolates of clinical origin nine (22.5%) were strongly positive for protease, 19 (47.5%) were positive and 12 (30.0%) were negative for protease. On the other hand, of the 44 isolates of environmental origin 20 (45.5%) were strongly positive for protease, 13 (29.5%) were positive for protease and 11 (25.0%) were negative for protease. No statistically differences were observed in the groups for protease.

Of the 40 isolates of clinical origin 37 (92.5%) were strongly positive, one (2.5%) was positive and two (5.0%) were negative for phospholipase (Table 1). Of the 44 isolates of environmental origin 24 (54.5%) were strongly positive and 20 (45.5%) were positive for phospholipase. The difference between the groups was statistically significant for phospholipase ($P = 0.0003$).

4.3. Susceptibility to Antifungals

Fluconazole—In isolates of clinical origin, the MIC values ranged from 0.0940 to 4.000 μg/mL, with a mean value of 0.8091 μg/mL. In isolates of environment origin, the values ranged from 0.0320 to 8.000 μg/mL, with a mean value of 0.6200 μg/mL.

Itraconazole—In isolates of clinical origin, the MIC values ranged from 0.0080 to 0.2500 μg/mL, with a mean value of 0.0661 μg/mL. In isolates of environment origin, the values ranged from 0.0080 to 0.5000 μg/mL, with a mean value of 0.0388 μg/mL.

Voriconazole—In isolates of clinical origin, the MIC values ranged from 0.0020 to 0.0320 μg/mL, with a mean value of 0.0065 μg/mL. In isolates of environment origin, the values ranged from 0.0020 to 0.0640 μg/mL, with a mean value of 0.0057 μg/mL.

Posaconazole—In isolates of clinical origin, the MIC values ranged from 0.0020 to 0.0940 μg/mL, with a mean value of 0.0214 μg/mL. In isolates of

Table 1. Production of protease and phospholipase by the isolates.

	C. neoformans	*C. neoformans*	*C. gatti*	*C. gatti*
	Clinical origin	Environmental origin	Clinical origin	Environmental origin
PROTEASE				
Strongly positive (%)	7 (19.4%)	18 (42.9%)	2 (50.0%)	2 (100%)
Positive (%)	18 (50.0%)	13 (31.0%)	1 (25.0%)	---
Negative (%)	11 (30.6%)	11 (26.1%)	1 (25.0%)	---
PHOSPOLIPASE				
Strongly positive (%)		22 (52.4%)	4 (100%)	2 (100%)
Positive (%)	2 (5.5%)	20 (47.6%)	---	---
Negative (%)	2 (5.5%)	---	---	---

environment origin, the values ranged from 0.0020 to 0.0640 μg/mL, with a mean value of 0.0178 μg/mL.

Amphotericin B—In isolates of clinical origin, the MIC values ranged from 0.0120 to 0.1250 μg/mL, with a mean value of 0.0490 μg/mL. Isolates of environmental origin presented MIC values ranging from 0.0020 to 0.1900 μg/mL, with a mean value of 0.0348 μg/mL.

5. Discussion

In this study all isolates of *C. neoformans* were retested in relation to biochemical tests and the six isolates, four from clinical origin and two from environmental origin, which were positive in CGB-positive, were submitted to molecular typing by RFLP-PCR. Only four presented a genomic profile consistent with the *C. gattii* species (VGII), while two other were, in fact, *C. neoformans* (VNI and VNIII).

Traditionally, *C. neoformans* has been grouped in three varieties: *C. neoformans* var. *gattii* (serotype B and C), *C. neoformans* var. *grubii (serotype A)* and *C. neoformans* var. *neoformans* (serotype D and AD) [37]. However, the *gattii* variety has been renamed as *C. gattii* [3] [38]. According this nomenclature, all our strains of *C. neoformans* were retested from the biochemical tests and the six strains that were positive in the CGB medium were then presumptively reclassified as *C. gattii* [3].

Recently, a new classification of *C. neoformans* complex has been proposed [39], adding new species, and the former species *C. neoformans* and *C. gattii* are now considered to be a complex of seven species: *C. neoformans* (former *C. neoformans* var. *grubii* genotypes VNI and VNII), *Cryptococcus deneoformans* (former *C. neoformans* var. *neoformans,* genotype VNIV), *C. gattii* (former *C. gattii* genotype VGI), *Cryptococcus bacillisporus* (former *C. gattii* genotype VGIII), *Cryptococcus deuterogattii* (former *C. gattii* genotype VGII), *Cryptococcus tetragattii* (former *C. gattii* VGIV), *Cryptococcus decagattii* (former *C. gattii* VGIV/VGIIIc), plus at least four hybrid of these species [39]. Considering this new classification, four strains of *C. gattii* of our stock could be named as *Cryptococcus deuterogattii* and one as *C. neoformans*.

In relation to the methods used in the phenotypic characterization, Klein *et al.* (2009) [8] analyzed 102 strains of yeast to verify urease activity, melanin production and glycine assimilation in the CGB medium as well as sequencing. The 17 *C. gattii* strains tested were positive in the CGB medium, the 54 *C. neoformans* strains were negative in the CGB medium, one of which had weak staining activity in the medium, six of 20 other *Cryptococcus* species were also positive in the CGB medium, but negative for melanin production. This demonstrates that there is a limitation in the exclusive use of CGB culture for identification of *Cryptococcus* species and suggests that all isolates, clinical or environmental should be tested by genomic methods such as PCR-RFLP to confirm their real taxonomic identity [8].

Indeed, in our study we observed that two strains of *C. neoformans* displayed urease activity which is not a common phenomenon. However, there are been some reports on urease activity by well characterized *C. neoformans* isolates [40] [41] [42]. The possible explanation is that these strains suffered some kind of mutations and became canavanine-resistant [40] [41] [42].

There are several published works that describe the importance of different extracellular enzymes, such as urease, protease, phospholipase and phenoloxidase, among others, that allow the yeast to alter pH in the phagolysosome, degrades proteins of importance for the immune response of the host, favoring invasion [23] [43]. In our study, most of the *Cryptococcus* strains, both of clinical origin and environmental origin, presented high protease values, although some of them, in the two groups, were not observed to produce significant amounts of this enzyme. This demonstrates the difficulty of correlating proteolytic activity observed by *in vitro* experiments with possible *in vivo* effects, for example, determining their actual contribution to disease development.

When the production of phospholipase was evaluated, it was observed that most of the isolates of clinical origin showed high production of the enzyme (92.5% were strongly positive and 2.5% positive), at a higher frequency than that observed in isolates of environmental origin (54.5% were strongly positive and 45.5% positive). Vidotto *et al.* (1996) [44] tested 23 isolates of *C. neoformans* for phospholipase production and found positivity in 95.7% (22) isolates, with a Pz ranged between 0.271 and 0.949.

According to the authors, there was a correlation between the phospholipase production and the size of the capsule in the isolated from AIDS patients. However, in this study, it was not possible to establish a relationship between capsular thickness and phospholipase production. In addition, genetic disruption studies have revealed the importance of the phospholipase B gene in yeast virulence in animal models, observing a decrease in the activity of this enzyme when the gene is altered [45] [46]. Thus, phospholipase activity would be a more useful phenotypic marker to determine and evaluate the potential degree of virulence among isolates from clinical and environmental isolates of *C. neoformans* complex [45] [46] [47].

Extracellular phospholipases are a heterogeneous group of enzymes produced by several bacteria and fungi and have been implicated in their pathogenicity because they can cause damage to host cell membranes that result in the destabilization of membranes, cell lysis and release of lipid second messengers [48] [49] [50]. Studies of *Candida albicans* demonstrated that phospholipase activity was correlated with mucosal invasion [51] and increased mortality in mice [52]. Also, production of phospholipases A, B, C and D by *Aspergillus fumigatus* has been reported [52] [53].

In relation to ecological aspects of *Cryptococcus* several studies suggest that species of the *C. neoformans/C. gattii* complex are interrelated epidemiologically and ecologically [5] [54]-[59]. *C. neoformans* is found in most cryptococcal infections worldwide, mainly in patients underlying immunocompromised condi-

tion as AIDS patients and presents the pigeon droppings and soil of as the main reservoir. In our study, the majority of isolates of environmental origin used came from pigeon droppings and other birds, where the most frequent species is *C. neoformans* [6] [59] [60] [61] [62]. Although *C. neoformans* and *C. gattii* occupy similar ecological niches, *C. gattii* is mainly associated with deteriorated wood, plant remains and soil. In particular, eucalyptus trees represent the most common substrate for *C. gattii* [62]-[67], and we could confirm this, as our samples isolated from *Eucalyptus* leaves belong to the *C. gattii* species.

Cryptococcal infections in humans usually are treated with amphotericin B, associated or not with 5-flucytosine, almost followed by maintenance therapy with some azole antifungal, mainly fluconazole. High rates of cryptococcosis recidiva suggest a potential emergence of antifungal resistance, clinical or otherwise, among *C. neoformans* isolates [68] [69] [70]. In view of this situation, it is necessary to routinely determine the sensitivity profile of *C. neoformans* isolates to different antifungal agents and, in addition, to allow an evaluation of the efficacy of the treatments used and the evolution of the disease.

In this present study, we used disk diffusion tests, the commercial Etest kit that has been described as a good method for routine laboratory use, proving to have a good alternative for *in vitro* sensitivity tests for azoles [71] [72] [73]. This method allows the determination of MICs, and has shown excellent benefits, such as easy execution and quick results [74]. It also demonstrates good correlation with the standard method by CLSI [35] which is more labor-intensive and a long reading time. Although CLSI methods are not considered ideal for testing *C. neoformans* [75] [76], broth microdilution and agar diffusion method have been applied worldwide to study the antifungal sensitivities of *C. neoformans* isolates.

Both clinical and environmental isolates were highly sensible to the antifungals amphotericin B, posaconazole and voriconazole. Several studies have reported that amphotericin B as well as new triazoles (voriconazole and posaconazole) has potent activity against *C. neoformans*, where more than 99% of the isolates show MICs ≤ 1.0000 μg/mL, regardless of geographic origin of isolates [77] [78] [79], suggering that this new triazoles may bealternative treatements when similar isolates are finded in clinical practice [79] [80].

A limitation of this study was that we did not genotype all samples nor could test the samples for their susceptibility to antifungals by the CLSI protocol, due to financial constraints. On the other hand, we were able to better characterize beyond a simple taxonomic identification the isolates of the *Cryptococcus* kept as stock at microrganism's bank of the University of São Paulo. We also could demonstrate the occurrence of variations on the samples such as more phospholipase activity in samples collected from clinical cases than in samples from environment, the presence of two canavanine-resistant *C. neoformans* strains and none of the samples were resistant to any antifungals tested. Therefore, the importance of maintaining a well characterized collection of *Cryptococcus* is that

one can be better assess their characteristics as new laboratory tools become available.

6. Conclusion

We point out in this research the existence of *C. neoformans* strains resistant to canavanine and intrinsic characteristic of *C. gatti*. The other important results obtained were the higher levels of phospholipase by clinical strains than environmental strains but no differences were observed for the protease levels. Selected phenotypic characteristics of *C. neoformans* and *C. gatti* complex must be compared to molecular studies to perform a correct diagnosis to the species.

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Conflict of Interest

All authors declared that there is no conflict of interest.

References

[1] Brandt, M.E., Hutwagner, L.C., Klug, L.A., Baughman, W.S., Rimland, D., Gravis, E.A., Hamill, R.J., Thomas, C., Pappas, P.G., Reingold, A.L. and Pinner, R.W. (1996) Molecular Subtype Distribution of *Cryptococcus neoformans* in Four Areas of the United States. Cryptococcal Disease Active Surveillance Group. *Journal of Clinical Microbiology*, **34**, 912-917.

[2] Iqbal, N., DeBess, E.E., Wohrle, R., Sun, B., Nett, R.J., Ahlquist, A.M., Chiller, T., Lockhart, S.R., *et al.* (2010) Correlation of Genotype and *In Vitro* Susceptibilities of *Cryptococcus gattii* Strains from the Pacific Northwest of the United States. *Journal of Clinical Microbiology*, **48**, 539-544. https://doi.org/10.1128/JCM.01505-09

[3] Kurtzman, C.P., Fell, J.W. and Boekhout, T. (2011) The Yeasts: A Taxonomic Study. 5th Edition, ACM Press, NY.

[4] Emmons, C.W. (1951) Isolation of *Cryptococcus neoformans* from Soil. *Journal of Bacteriology*, **62**, 685-690.

[5] Emmons, C.W. (1955) Saprophytic Sources of *Cryptococcus neoformans* Associated with the Pigeon (*Columba livia*). *American Journal of Hygiene*, **62**, 227-232. https://doi.org/10.1093/oxfordjournals.aje.a119775

[6] Baroni, F.A., Paula, C.R., Silva, E.G., Viani, F.C., Rivera, I.N., Oliveira, M.T.B. and Gabale, W. (2006) *Cryptococcus neoformans* Strains Isolated from Church Towers in Rio de Janeiro City, RJ, Brazil. *The Revista do Instituto de Medicina Tropical de São Paulo*, **48**, 71-75. https://doi.org/10.1590/S0036-46652006000200003

[7] Lin, X. and Heitman, J. (2006) The Biology of the *Cryptococcus neoformans* Species Complex. *Annual Review of Microbiology*, **60**, 69-105. https://doi.org/10.1146/annurev.micro.60.080805.142102

[8] Klein, K.R., Hall, L., Deml, S.M., Rysavy, J.M., Wohlfiel, S.L. and Wengenack, N.L.

(2009) Identification of *Cryptococcus gattii* by Use of L-Canavanine Glycine Bromothymol Blue Medium and DNA Sequencing. *Journal of Clinical Microbiology*, **47**, 3669-3672. https://doi.org/10.1128/JCM.01072-09

[9] Kwon-Chung, K.J., Polacheck, I. and Bennett, J.E. (1982) Improved Diagnostic Medium for Separation of *Cryptococcus neoformans* var. *neoformans* (Serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (Serotypes B and C). *Journal of Clinical Microbiology*, **15**, 535-537.

[10] Ellis, D., Marriott, D., Hajjeh, R.A., Warnock, D., Meyer, W. and Barton, R. (2000) Epidemiology: Surveillance of Fungal Infections. *Medical Mycology*, **38**, 173-182. https://doi.org/10.1080/mmy.38.s1.173.182

[11] Meyer, W., Castañeda, A., Jackson, S., Huynh, M., Castañeda, E., *et al.* (2003) Molecular Typing of IberoAmerican *Cryptococcus neoformans* Isolates. *Emerging Infectious Diseases*, **9**, 189-195. https://doi.org/10.3201/eid0902.020246

[12] Silva, D.C., Martins, M.A., Szeszs, M.W., Bonfietti, L.X., Matos, D. and Melhem, M.S. (2012) Susceptibility to Antifungal Agents and Genotypes of Brazilian Clinical and Environmental *Cryptococcus gattii* Strains. *Diagnostic Microbiology and Infectious Disease*, **72**, 332-339. https://doi.org/10.1016/j.diagmicrobio.2011.11.016

[13] Diaz, M.R., Boekhout, T., Kiesling, T. and Fell, J.W. (2005) Comparative Analysis of the Intergenic Spacer Regions and Population Structure of the Species Complex of the Pathogenic Yeast *Cryptococcus neoformans. FEMS Yeast Research*, **5**, 1129-1140. https://doi.org/10.1016/j.femsyr.2005.05.005

[14] Boekhout, T., Theelen, B., Diaz, M., Fell, J.W., Hop, W.C., Abeln, E.C., Dromer, F. and Meyer, W. (2001) Hybrid Genotypes in the Pathogenic Yeast *Cryptococcus neoformans. Microbiology*, **147**, 891-907. https://doi.org/10.1099/00221287-147-4-891

[15] Kidd, S.E., Guo, H., Bartlett, K.H., Xu, J. and Kronstad, J.W. (2005) Comparative Gene Genealogies Indicate that Two Clonal Lineages of *Cryptococcus gattii* in British Columbia Resemble Strains from Other Geographical Areas. *Eukaryotic Cell*, **4**, 1629-1638. https://doi.org/10.1128/EC.4.10.1629-1638.2005

[16] Fraser, J.A., Giles, S.S., Wenink, E.C., Geunes-Boyer, S.G., Wright, J.R., Diezmann, S., Allen, A., Stajich, J.E., Dietrich, F.S., Perfect, J.R. and Heitman, J. (2005) Same-Sex Mating and the Origin of the Vancouver Island *Cryptococcus gattii* Outbreak. *Nature*, **437**, 1360-1364. https://doi.org/10.1038/nature04220

[17] Steenbergen, J.N. and Casadevall, A. (2003) The Origin and Maintenance of Virulence for the Human Pathogenic Fungus *Cryptococcus neoformans. Microbes and Infection*, **5**, 667-675. https://doi.org/10.1016/S1286-4579(03)00092-3

[18] Chang, Y.C. and Kwon-Chung, K.J. (1994) Complementation of a Capsule-Deficient Mutation of *Cryptococcus neoformans* Restores Its Virulence. *Molecular and Cellular Biology*, **14**, 4912-4919. https://doi.org/10.1128/MCB.14.7.4912

[19] Rivera, J., Feldmesser, M., Cammer, M. and Casadevall, A. (1998) Organ-Dependent Variation of Capsule Thickness in *Cryptococcus neoformans* during Experimental Murine Infection. *Infection and Immunity*, **66**, 5027-5030.

[20] Zaragoza, O., Rodrigues, M.L., De Jesus, M., Frases, S., Dadachova, E. and Casadevall, A. (2009) The Capsule of the Fungal Pathogen *Cryptococcus neoformans. Advances in Applied Microbiology*, **68**, 133-216. https://doi.org/10.1016/S0065-2164(09)01204-0

[21] Wang, Y., Aisen, P. and Casadevall, A. (1995) *Cryptococcus neoformans* Melanin and Virulence: Mechanism of Action. *Infection and Immunity*, **63**, 3131-3136.

[22] Cox, G.M., Mukherjee, J., Cole, G.T., Casadevall, A. and Perfect, J.R. (2000) Urease as a Virulence Factor in Experimental Cryptococcosis. *Infection and Immunity*, **68**, 443-448. https://doi.org/10.1128/IAI.68.2.443-448.2000

[23] Cox, G.M., McDade, H.C., Chen, S.C., Tucker, S.C., Gottfredsson, M., Wright, L.C., Sorrell, T.C., Leidich, S.D., Casadevall, A., Ghannoum, M.A. and Perfect, J.R. (2001) Extracellular Phospholipase Activity Is a Virulence Factor for *Cryptococcus neoformans*. *Molecular Microbiology*, **39**, 166-175.

[24] Liu, G.Y. and Nizet, V. (2009) Color Me Bad: Microbial Pigments as Virulence Factors. *Trends in Microbiology*, **17**, 406-413.

[25] Moraes, E.M., Prímola, N.S. and Hamdan, J.S. (2003) Antifungal Susceptibility of Clinical and Environmental Isolates of *Cryptococcus neoformans* to Four Antifungal Drugs Determined by Two Techniques. *Mycoses*, **46**, 164-168. https://doi.org/10.1046/j.1439-0507.2003.00872.x

[26] Souza, L.K., Fernandes, O.F., Kobayashi, C.C., Passos, X.S., Costa, C.R., Lemos, J.A, Souza-Jr., H.S. and Silva, M.R.R. (2005) Antifungal Susceptibilities of Clinical and Environmental Isolates of *Cryptococcus neoformans* in Goiânia City, Goiás, Brazil. *The Revista do Instituto de Medicina Tropical de São Paulo*, **47**, 253-256. https://doi.org/10.1590/S0036-46652005000500003

[27] Chowdhary, A., Randhawa, H.S., Sundar, G., Kathuria, S., Prakash, A., Khan, Z, Sun, S. and Xu, J. (2011) *In Vitro* Antifungal Susceptibility Profiles and Genotypes of 308 Clinical and Environmental Isolates of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* Serotype B from Northwestern India. *Journal of Medical Microbiology*, **60**, 961-967. https://doi.org/10.1099/jmm.0.029025-0

[28] Martins, M.A., Pappalardo, M.C., Melhem, M.S. and Pereira-Chioccola, V.L. (2007) Molecular Diversity of Serial *Cryptococcus neoformans* Isolates from AIDS Patients in the City of São Paulo, Brazil. *The Memórias do Instituto Oswaldo Cruz*, **102**, 777-784. https://doi.org/10.1590/S0074-02762007000700001

[29] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. 2nd Edition, Cold Spring Harbor Laboratory Press, New York.

[30] Macdougall, L., Kidd, S.E., Galanis, E., Mak, S., Leslie, M.J., Cieslak, P.R., Kronstad, J.W., Morsehed, M.G. and Bartlett, K.H. (2007) Spread of *Cryptococcus gattii* in British Columbia, Canada, and Detection in the Pacific Northwest, USA. *Emergent Infectious Diseases Journal*, **13**, 42-50. https://doi.org/10.3201/eid1301.060827

[31] Rüchel, R., Tegeler, R. and Trost, M. (1982) A Comparison of Secretory Proteinases from Different Strains of *Candida albicans*. *Sabouraudia*, **20**, 233-244. https://doi.org/10.1080/00362178285380341

[32] Price, M.F., Wilkinson, I.D. and Gentry, L.O. (1982) Plate Method for Detection of Phospholipase Activity in *Candida albicans*. *Sabouraudia*, **20**, 7-14. https://doi.org/10.1080/00362178285380031

[33] Lozano-Chiu, M., Paetznick, V.L., Ghannoum, M.A. and Rex, J.H. (1998) Detection of Resistance to Amphotericin B among *Cryptococcus neoformans* Clinical Isolates: Performances of Three Different Media Assessed by Using E-Test and National Committee for Clinical Laboratory Standards M27-A Methodologies. *Journal of Clinical Microbiology*, **36**, 2817-2822.

[34] Pfaller, M.A., Messer, S.A., Houston, A., Mills, K., Bolmstrom, A. and Jones, R.N. (2000) Evaluation of the Etest Method for Determining Voriconazole Susceptibilities of 312 Clinical Isolates of *Candida* Species by Using Three Different Agar Media. *Journal of Clinical Microbiology*, **38**, 3715-3717.

[35] Clinical and Laboratory Standards Institute (2012) Reference Method for Broth Di-

lution Antifungal Susceptibility Testing of Yeasts. 4th Informational Supplement, CLSI Document M27-S4, Clinical and Laboratory Standards Institute, Wayne, PA.

[36] Kumar, D., Bhattacharyya, S., Gupta, P., Banerjee, G. and Singh, M. (2015) Comparative Analysis of Disc Diffusion and E-Test with Broth Micro-Dilution for Susceptibility Testing of Clinical *Candida* Isolates against Amphotericin B, Fluconazole, Voriconazole and Caspofungin. *Journal of Clinical and Diagnostic Research*, **9**, DC01-DC04.

[37] Franzot, S.P., Salkin, I.F. and Casadevall, A. (1999) *Cryptococcus neoformans* var. *grubii*: Separate Varietal Status for *Cryptococcus neoformans* Serotype A Isolates. *Journal of Clinical Microbiology*, **37**, 838-840.

[38] Heitman, J. (2011) Microbial Pathogens in the Fungal Kingdom. *Fungal Biology Reviews*, **25**, 48-60. https://doi.org/10.1016/j.fbr.2011.01.003

[39] Hagen, F., Khayhan, K., Theelen, B., Kolecka, A., Polacheck, I., Sionov, E., Falk, R., Parnmen, S., Lumbsch, H.T. and Boekhout, T. (2015) Recognition of Seven Species in the *Cryptococcus gattii*/*Cryptococcus neoformans* Species Complex. *Fungal Genetics and Biology*, **78**, 16-48. https://doi.org/10.1016/j.fgb.2015.02.009

[40] Polacheck, I. and Kwon-Chung, K.J. (1986) Canavanine Resistance in *Cryptococcus neoformans*. *Antimicrobial Agents and Chemotherapy*, **29**, 468-473. https://doi.org/10.1128/AAC.29.3.468

[41] Nakamura, Y., Kano, R., Sato, H., Watanabe, S., Takahashi, H. and Hasegawa, A. (1998) Isolates of *Cryptococcus neoformans* Serotype A and D Developed on Canavanine-Glycine-Bromthymol Blue Medium. *Mycoses*, **41**, 35-40.

[42] Khan, Z.U., Al-Anezi, A.A., Chandy, R. and Xu, J. (2003) Disseminated Cryptococcosis in an AIDS Patient Caused by A Canavanine-Resistant Strain of *Cryptococcus neoformans* var. *grubii*. *Journal of Medical Microbiology*, **52**, 271-275. https://doi.org/10.1099/jmm.0.05097-0

[43] Casadevall, A. and Perfect, J.R. (1998) *Cryptococcus neoformans*. ASM Press, Washington DC.

[44] Vidotto, V., Sinicco, A., Di Fraia, D., Cardaropoli, S., Aoki, S. and Ito-Kuwa, S. (1996) Phospholipase Activity in *Cryptococcus neoformans*. *Mycopathologia*, **136**, 119-123. https://doi.org/10.1007/BF00438916

[45] Chen, S.C., Muller, M., Zhou, J.Z., Wright, L.C. and Sorrell, T.C. (1997) Phospholipase Activity in *Cryptococcus neoformans*: A New Virulence Factor? *The Journal of Infectious Diseases*, **175**, 414-420. https://doi.org/10.1093/infdis/175.2.414

[46] Titball, R.W. (1993) Bacterial Phospholipases C. *Microbiological Reviews*, **57**, 347-366.

[47] Cardoso, P.H.M., dos Santos, R.L.O., Souza, A.C., Baroni, F.A., Domaneschi, C. and Paula, C.R. (2018) Biosynthesis of Phospholipases and Proteinases Using Clinical and Environmental Strains of *Cryptococcus neoformans*. *Clinical and Laboratorial Research in Dentistry*, **1**, 1-5. http://dx.doi.org/10.11606/issn.2357-8041.clrd.2018.133520

[48] Ghannoum, M.A. (2000) Potential Role of Phospholipases in Virulence and Fungal Pathogenesis. *Clinical Microbiology Reviews*, **13**, 122-143. https://doi.org/10.1128/CMR.13.1.122-143.2000

[49] Schmiel, D.H. and Miller, V.L. (1999) Bacterial Phospholipases and Pathogenesis. *Microbes and Infection*, **1**, 1103-1112. https://doi.org/10.1016/S1286-4579(99)00205-1

[50] Barrett-Bee, K., Hayes, Y., Wilson, R.G. and Ryley, J.F. (1985) A Comparison of

Phospholipase Activity, Cellular Adherence and Pathogenicity of Yeasts. *Journal of General Microbiology*, **131**, 1217-1221. https://doi.org/10.1099/00221287-131-5-1217

[51] Ibrahim, A.S., Mirbod, F., Filler, S.G., Banno, Y., Cole, G.T., Kitajima, Y., Edwards-Jr., J.E., Nozawa, Y. and Ghannoum, M.A. (1995) Evidence Implicating Phospholipase as a Virulence Factor of *Candida albicans*. *Infection and Immunity*, **63**, 1993-1998.

[52] Birch, M., Robson, G., Law, D. and Denning, D.W. (1996) Evidence of Multiple Extracellular Phospholipase Activities of *Aspergillus fumigatus*. *Infection and Immunity*, **64**, 751-755.

[53] Levitz, S.M. (1991) The Ecology of *Cryptococcus neoformans* and the Epidemiology of Cryptococcosis. *Reviews of Infectious Diseases*, **13**, 1163-1169.

[54] Li, A., Nishimura, K., Taguchi, H., Tanaka, R., Wu, S. and Miyaji, M. (1993) The Isolation of *Cryptococcus neoformans* from Pigeon Droppings and Serotyping of Naturally and Clinically Sourced Isolates in China. *Mycopathologia*, **124**, 1-5. https://doi.org/10.1007/BF01103049

[55] Pappalardo, M.C. and Melhem, M.S. (2003) Cryptococcosis: A Review of the Brazilian Experience for the Disease. *The Revista do Instituto de Medicina Tropical de São Paulo*, **45**, 299-305. https://doi.org/10.1590/S0036-46652003000600001

[56] Soares, M.C., Paula, C.R., Dias, A.L., Caseiro, M.M. and Costa, S.O.P. (2005) Environmental Strains of *Cryptococcus neoformans* Variety *grubii* in the City of Santos, SP, Brazil. *The Revista do Instituto de Medicina Tropical de São Paulo*, **47**, 31-36. https://doi.org/10.1590/S0036-46652005000100006

[57] Kwon-Chung, K.J., Fraser, J.A., Doering, T.L., Wang, Z., Idnurm, A. and Bahn, Y.S. (2014) *Cryptococcus neoformans* and *Cryptococcus gattii*, the Etiologic Agents of Cryptococcosis. *Cold Spring Harbor Perspectives in Medicine*, **4**, a019760. https://doi.org/10.1101/cshperspect.a019760

[58] Ajello, L. (1958) Ocurrence of *Cryptococcus neoformans* in Solis. *American Journal of Hygiene*, **67**, 72-77.

[59] Lazera, M.S., Salmito Cavalcanti, M.A., Londero, A.T., Trilles, L., Nishikawa, M.M. and Wanke, B. (2000) Possible Primary Ecological Niche of *Cryptococcus neoformans*. *Medical Mycology*, **38**, 379-383. https://doi.org/10.1080/mmy.38.5.379.383

[60] Escandón, P., Sánchez, A., Martínez, M., Meyer, W. and Castañeda, E. (2006) Molecular Epidemiology of Clinical and Environmental Isolates of the *Cryptococcus neoformans* Species Complex Reveals a High Genetic Diversity and the Presence of the Molecular Type VGII Mating Type an in Colombia. *FEMS Yeast Research*, **6**, 625-635. https://doi.org/10.1111/j.1567-1364.2006.00055.x

[61] Refojo, N., Perrotta, D., Brudny, M., Abrantes, R., Hevia, A.I. and Davel, G. (2009) Isolation of *Cryptococcus neoformans* and *Cryptococcus gattii* from Trunk Hollows of Living Trees in Buenos Aires City, Argentina. *Medical Mycology*, **47**, 177-184. https://doi.org/10.1080/13693780802227290

[62] Ellis, D.H. and Pfeiffer, T.J. (1990) Natural Habitat of *Cryptococcus neoformans* var. *gattii*. *Journal of Clinical Microbiology*, **28**, 1642-1644.

[63] Montenegro, H. and Paula, C.R. (2000) Environmental Isolation of *Cryptococcus neoformans* var. *gattii* and *C. neoformans* var. *neoformans* in the City of São Paulo, Brazil. *Medical Mycology*, **38**, 385-390. https://doi.org/10.1080/mmy.38.5.385.390

[64] Casali, A.K., Goulart, L., Rosa e Silva, L.K., Ribeiro, A.M., Amaral, A.A., Alves, S.H., Schrank, A., Meyer, W. and Vainsteins, M.H. (2003) Molecular Typing of Clinical and Environmental *Cryptococcus neoformans* Isolates in the Brazilian State Rio

Grande do Sul. *FEMS Yeast Research*, **3**, 405-415. https://doi.org/10.1016/S1567-1356(03)00038-2

[65] Granados, D.P. and Castañeda, E. (2006) Influence of Climatic Conditions on the Isolation of Members of the *Cryptococcus neoformans* Species Complex from Trees in Colombia from 1992-2004. *FEMS Yeast Research*, **6**, 636-644. https://doi.org/10.1111/j.1567-1364.2006.00090.x

[66] Mitchell, T.G., Castaneda, E., Niersen, K. and Wanke, B.M.S.L. (2011) Environmental Niches for *Cryptococcus neoformans* and *Cryptococcus gattii*. In: Heitman, J., *et al.*, Eds., *Cryptococcus*: *From Human Pathogen to Model Yeast*, ASM Press, Washington DC, 237-259.

[67] Berg, J., Clancy, C.J. and Nguyen, M.H. (1998) The Hidden Danger of Primary Fluconazole Prophylaxis for Patients with AIDS. *Clinical Infectious Diseases*, **26**, 186-187. https://doi.org/10.1086/517056

[68] Brandt, M.E., Pfaller, M.A., Hajjeh, R.A., Hamill, R.J., Pappas, P.G., Reingold, A.L., Rimland, D. and Warnock, D.W. (2001) Cryptococcal Disease Active Surveillance Group. Trends in Antifungal Drug Susceptibility of *Cryptococcus neoformans* Isolates in the United States: 1992 to 1994 and 1996 to 1998. *Antimicrobial Agents and Chemotherapy*, **45**, 3065-3069. https://doi.org/10.1128/AAC.45.11.3065-3069.2001

[69] Dias, A.L., Matsumoto, F.E., Melhem, M.S., Silva, E.G., Auler, M.E., Siqueira, A.M. and Paula, C.R. (2006) Comparative Analysis of Etest and Broth Microdilution Method (AFST-EUCAST) for Trends in Antifungal Drug Susceptibility Testing of Brazilian *Cryptococcus neoformans* Isolates. *Journal of Medical Microbiology*, **55**, 1693-1699. https://doi.org/10.1099/jmm.0.46789-0

[70] Matsumoto, F.E., Dias, A.L., Melhem, M.S., Szeszs, M.W., Auler, M.E., Ruiz, L.S., Gonçalves da Silva, E., Gandra, R.F. and Paula, C.R. (2007) Antifungal Susceptibility of Bloodstream Yeasts Isolated at a Public Children's Hospital in Brazil: Comparison of the Etest® and the AFST-EUCAST Microdilution Method. *Canadian Journal of Microbiology*, **53**, 1300-1306. https://doi.org/10.1139/W07-095

[71] Tewari, A., Behera, B., Mathur, P. and Xess, I. (2012) Comparative Analysis of the Vitek 2 Antifungal Susceptibility System and E-Test with the CLSI M27-A3 Broth Microdilution Method for Susceptibility Testing of Indian Clinical Isolates of *Cryptococcus neoformans*. *Mycopathologia*, **173**, 427-433. https://doi.org/10.1007/s11046-012-9528-9

[72] Mahabeer, Y., Chang, C.C., Naidu, D., Dorasamy, A., Lewin, S., Ndung'u, T., Moosa, M.Y., French, M., Mlisana, K. and Coovadia, Y. (2014) Comparison of Etests and Vitek 2® to Broth Microdilution for the Susceptibility Testing of *Cryptococcus neoformans*. *Diagnostic Microbiology and Infectious Disease*, **80**, 294-298. https://doi.org/10.1016/j.diagmicrobio.2014.09.006

[73] Negri, M., Henriques, M., Svidzinski, T.I., Paula, C.R. and Oliveira, R. (2009) Correlation between Etest, Disk Diffusion, and Microdilution Methods for Antifungal Susceptibility Testing of *Candida* Species from Infection and Colonization. *Journal of Clinical Laboratory Analysis*, **23**, 324-330. https://doi.org/10.1002/jcla.20337

[74] Ghannoum, M.A., Ibrahim, A.S., Fu, Y., Shafig, M.C., Edwards Jr., J.E. and Criddle, R.S. (1992) Susceptibility Testing of *Cryptococcus neoformans*: A Microdilution Technique. *Journal of Clinical Microbiology*, **30**, 2881-2886.

[75] Witt, M.D., Lewis, R.J., Larsen, R.A., Milefchik, E.N., Leal, M.A., Haubrich, R.H., Richie, J.A., Edwards Jr., J.E. and Ghannoum, M.A. (1996) Identification of Patients with Acute AIDS-Associated Cryptococcal Meningitis who Can Be Effectively Treated with Fluconazole: The Role of Antifungal Susceptibility Testing. *Clinical*

Infectious Diseases, **22**, 322-328. https://doi.org/10.1093/clinids/22.2.322

[76] Alves, S.H., Oliveira, L.T., Costa, J.M., Lubeck, I., Casali, A.K. and Vainstein, M.H. (2001) *In Vitro* Susceptibility to Antifungal Agents of Clinical and Environmental *Cryptococcus neoformans* Isolated in Southern of Brazil. *The Revista do Instituto de Medicina Tropical de São Paulo*, **43**, 267-270. https://doi.org/10.1590/S0036-46652001000500006

[77] Bii, C.C., Makimura, K., Abe, S., Taguchi, H., Mugasia, O.M., Revathi, G., Wamae, N.C. and Kamiya, S. (2007) Antifungal Drug Susceptibility of *Cryptococcus neoformans* from Clinical Sources in Nairobi, Kenya. *Mycoses*, **50**, 25-30. https://doi.org/10.1111/j.1439-0507.2006.01293.x

[78] Capoor, M.R., Mandal, P., Deb, M., Aggarwal, P. and Banerjee, U. (2008) Current Scenario of Cryptococcosis and Antifungal Susceptibility Pattern in India: A Cause for Reappraisal. *Mycoses*, **51**, 258-265. https://doi.org/10.1111/j.1439-0507.2007.01478.x

[79] Thompson, G.R., Wiederhold, N.P., Fothergill, A.W., Vallor, A.C., Wickes, B.L. and Petterson, T.F. (2009) Antifungal Susceptibilities among Different Serotypes of *Cryptococcus gattii* and *Cryptococcus neoformans*. *Antimicrobial Agents and Chemotherapy*, **53**, 309-311. https://doi.org/10.1128/AAC.01216-08

[80] Tomazin, R., Matos, T., Meis, J.F. and Hagen, F. (2018) Molecular Characterization and Antifungal Susceptibility Testing of Sequentially Obtained Clinical *Cryptococcus deneoformans* and *Cryptococcus neoformans* Isolates from Ljubljana, Slovenia. *Mycopathologia*, **183**, 371-380. https://doi.org/10.1007/s11046-017-0214-9