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CLINICAL MYCOLOGY LAB ISSUES (S CORDOBA, SECTION EDITOR)

Usefulness of Yeast Cell Counting and Lack of Clinical Correlation of the Antifungal Susceptibility Testing Results in Management of Aids-associated Cryptococcal Meningitis

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Abstract

Purpose of Review Cryptococcal meningitis is one of the most seriously opportunistic infections in people living with HIV. We evaluated clinical and laboratorial features (minimum inhibitory concentrations for fluconazole, initial fungal burden in cerebrospinal fluid) and risk factors associated with in-hospital mortality.

Recent Findings There is no good evidence for the use of minimum inhibitory concentrations for fluconazole in routine practice for the management of cryptococcosis patients. Counting yeast cells at cerebrospinal fluid can predict positive culture by not death.

Summary Data from 46 cryptococcal meningitis patients were reviewed, retrospectively. Patients who presented yeast cell count greater than 400 yeast cells/μ in their initial cerebrospinal fluid sample were associated with higher mortality ($p = 0.014$); moreover, the yeast cell count is an easy and cheap assay, with high values possibly associated to poor prognosis. Additionally, we verified no significant differences between fluconazole susceptibility profile, molecular type, clinical presentation, cytological analyses, time to sterilize the cerebrospinal fluid, agent recovering out of central nervous system, previous diagnosis of cryptococcal meningitis or usage of fluconazole, and overall mortality.

Keywords Cryptococcal meningitis · Yeast cell count · Antifungal agents · Aids-related opportunistic infection · Outcome

Introduction

Cryptococcocal meningitis (CM) is an invasive fungal disease with a high global burden, especially in developing countries. It is the main cause of meningitis in sub-Saharan Africa in person living with HIV, despite antiretroviral therapy expansion [1, 2].

Cryptococcosis in aids is mostly caused by *C. neoformans* var. *grubii* (molecular type VNI and VNII) or *C. neoformans* (VNIII and VNIV types), and *C. gattii* (VGI, VGII, VGIII, and VGIV) is prevalent in apparently immunocompetent hosts [3•]. Otherwise, Hagen and coworkers proposed 7 species base in the phenotypic heterogeneity of *C. neoformans*/C. *gattii* complex [4•, 5•].

Data from 2014 estimates a range of 150,600 to 282,400 CM cases/year worldwide, most of them in sub-Saharan Africa (73%) with an estimated number of deaths/year ranging from 119,400 to 234,300 [6•]. The mortality in North America ranges from 9 to 20%, whereas in sub-Saharan Africa, it can reach 70% [7–10]. Brazilian epidemiological data are scarce, and although recent studies have shown a consistent decline in cases of extrapulmonary cryptococcosis, the mortality rates are still high, with 40% in São Paulo state and up to 32% observed at reference centers [11–13].

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World Health Organization (WHO) estates the treatment of CM cases should be 1 week of induction phase with amphotericin B (AMB) and 5-flucytosine (5FC) followed by 1 week of high doses of fluconazole (FLU) (1200 mg/day) [14•]. Other authors defend a more aggressive treatment and the induction phase should be with AMB and 5FC for 2 weeks, followed by FLU (800 mg/day) for 8 weeks [15•]. In Brazil, the treatment induction phase involves AMB along with FLU due to the difficulty of obtaining 5FC. FLU is considered to be an alternative to 5FC at the induction phase and is the drug of choice for the maintenance phase [15•]. Over the last decade, clinicians have been concerned about the rates of persistent disease during treatment and relapse [16–18], thus evoking great concern in monitoring the resistance against FLU among the cryptococcosis etiological agents.

Susceptibility testing is supposed to predict the reliability of clinical outcomes when an infected patient is treated with the specific agent and is referred to as the clinical breakpoint (CBP) [19, 20], which is based on minimum inhibitory concentrations (MIC), pharmacokinetic and pharmacodynamic (PK/PD) parameters, animal studies, and clinical outcomes. The epidemiological cutoff (ECV) is based mostly on MIC distributions, which comprise wild-type and non-wild-type populations; the ECV is the highest MIC that occurs in the wild-type population. While CBPs can predict the clinical outcome, the role of the ECV is to detect both emerging resistance and non-wild-type strains with reduced susceptibility (encompassing mutations) [16, 21–24, 25•, 26, 27••]. In this scenario, susceptibility testing for *Cryptococcus* spp. is still controversial due to limited clinical evidence of therapeutic failure and higher MICs for FLU [28•, 29, 30•]. We evaluate the clinical and laboratory findings of Brazilian CM cases associated with the MICs results for FLU, and additionally we assessed risk factors associated with in-hospital mortality aiming to outline clinical–laboratory tools that indicate a worse outcome, to alert clinicians to implementation of efficient strategies involved at CM management.

A retrospective cohort was assessed at a tertiary public hospital and reference center for infectious diseases located in São Paulo city (Instituto de Infectologia Emílio Ribas–IIER).

The study involved 46 aids patients under induction therapy for CM whose cerebrospinal fluid (CSF) samples were sent randomly by laboratory technician to our reference public laboratory (Instituto Adolfo Lutz–IAL), and were viable for the study.

The induction phase was defined as AMB usage combined with either FLU or 5FC. Treatments were considered successful when patients had improved signs and symptoms and presented with uninfected CSF. Treatment failure was defined when patients presented with either persistent signs and symptoms of infection or a positive culture after 4 weeks of treatment.

Increased intracranial pressure (ICP) was defined as an elevated opening pressure (OP) ≥ 25 cm H_2O [31]. Data regarding HIV status, such as CD4 lymphocytes and viral load, were collected. We also evaluated the presence of non-CNS *Cryptococcus* infection, previous CM infection, and use of FLU.

CSF cytological and biochemical analyses, yeast cell counting for assessment of initial fungal burden, and the time required for CSF sterilization were evaluated. Quantitative CSF yeast cell counts were performed by using a direct microscopic technique with a Fuchs–Rosenthal cell counting chamber [32••]. Isolates of *Cryptococcus* spp. were characterized at the species and molecular levels [3•, 33, 34] as well as by mating type [35]. Molecular-type standard strains were WM 148 (VNI), WM 626 (VNII), WM 628 (VNII), 629 (VNIV), WM 179 (VGI), WM 178 (VGII), WM 161 (VGIII), and WM 779 (VGIV). *Filobasidiella neoformans* ATCC 28957 (mating type alpha) and ATCC 28958 (mating-type a) were used as control strains.

The in vitro susceptibility profiles of the etiological agents against FLU and AMB were determined using the broth microdilution method M27-A3 (CLSI, 2008). We categorized MICs according to previously proposed FLU ECVs of 8 μg/L for *C. neoformans* VNI and 16 μg/mL for non-typed *C. neoformans* [27••]. Because data for defining the ECV for VNII were insufficient, we used the value suggested for non-typed *C. neoformans.* Regarding AMB, ECVs of 0.5 μg/mL for *C. neoformans* VNI and 1 μg/mL for non-typed *C. neoformans* were proposed and adopted in this study [36].

The correlations among clinical and laboratory data with the MICs were assessed. Moreover, we evaluated the MICs and other factors associated with global in-hospital mortality. The association between exposure and outcome was estimated by the odds ratio (OR). Additionally, the confidence interval (CI) for the bivariate relationship between the variables and death was estimated. In this study, we adopted a significance level of 5%.

The baseline number of initial *Cryptococcus* cells in the CSF was categorized using the receiver operating characteristic (ROC) curve to determine the most accurate variable associated with the outcome as defined by the area under the curve. The cutoff was determined by the greatest value from the sum of the sensitivity and specificity, which corresponds to the point of maximum inflection of the ROC curve. For the survival analysis, we used Kaplan–Meier curves with a log-rank test.

For qualitative variables, the results are presented as frequencies, and the quantitative estimates were reported as measures of central tendency and dispersion. The chi-square test was used for categorical variables. After verifying non-normality by the Shapiro–Wilk test, the nonparametric Mann–Whitney test was used to compare quantitative variables.

The following databases and software were used: Excel (for data entry) and IBM SPSS (Statistical Package for the Social Sciences) V21.0.

Clinical and laboratory characteristics of 46 CM patients were depicted in Table 1. Out of 46 patients, only 26% had recent HIV diagnoses, and 84% did not use antiretroviral therapy properly. At the time of admission, all of the patients presented with CNS symptoms of CM with a median disease duration of 15 (2–120) days. Severe neurological symptoms were less common and manifested as an altered mental status (26%), seizures (8%), and motor deficit (4%). Seventy-six percent of patients presented with ICP and 24% received a shunt. A median of 16 lumbar punctures (range, 0–31) were performed before the neurosurgery when this intervention was indicated.

Combination therapy with AMB and 5FC was administered to 26% of the patients, and AMB plus FLU was administered to 74% of the patients. The median time of hospital stay was 36 (range, 9–202) days, and the median induction phase was 28 (range, 1–92) days.

The cryptococcal latex agglutination test was positive in all of the patients, but the quantification was not performed by our service during the study period. Sterilization of the CSF occurred in 69% of patients after a median of 14 (range, 2–62) days. Statistical association between mortality and patients who did not achieve sterile CSF culture during hospitalization was observed (OR =

Table 1 Clinical and laboratory characteristics from patients infected with HIV and cryptococcal meningitis

Clinical and laboratory characteristics	Value
Sex—*n* (%)	
Male	34 (73.9)
Female	12 (26.1)
Age—mean (±sd)	39.7 years (± 11.09)
CD4 lymphocyte count (cells/mm^3)—median (range)	39 (2–196)
Viral load (copies/mL) —median (range)	90,496 (0–1,114,953)
CNS symptoms—*n* (%)	
Headache	42(91.3)
Nausea/vomiting	25(54.3)
Fever	25(54.3)
Concomitant opportunistic infection—*n* (%)	19 (41.3)
Oropharyngeal candidiasis	14 (30.4)
Tuberculosis	6 (13)
Cytological characteristics of CSF—median	
Leukocytes (cells/mm^3)	8.0
Proteins (g/L)	54
Glucose (mg/dL)	41.5
Increased intracranial pressure—*n* (%), *missing 4	
≥ 25 cmH2O	32 (76)
< 25 cmH2O	10 (24)
Initial count of yeasts (cells/μ) —median (range)	400 (0–11,520)
Cryptococcus out of CNS—*n* (%)	12 (26)
Blood	9 (19.7)
Skin	1 (2.1)
Lymph nodes	1 (2.1)
Bone marrow	1 (2.1)
Molecular type—*n* patients (%), *missing 1	
VNI	39 (86.6)
VNII	6 (13.4)
Outcome—*n* (%)	
Died	16 (34.8)
Discharge	28 (60.8)
Left	2 (4.4)

42.00, 95% CI, 6.75–261.07; $p \leq 0.001$). Of note, 4 patients presented with an uninfected CSF culture only after 4 weeks (range, 41–62 days) during the induction phase treatment.

The median fungal burden was 400 (range, 0–11,520) cells/μL in the first sample. Furthermore, analyses of the initial CSF fungal burden and in-hospital deaths demonstrated a cutoff value of 400 yeast cells/μL, with the ROC curve and the area under the curve equal to 0.81 and a significant difference compared to the null line ($p = 0.001$). Thus, patients who presented an initial CSF fungal burden greater than 400 yeast cells/μL had a higher mortality compared with patients who presented a fungal burden ≤ 400 (OR = 11.14; 95% IC, 2.41–51.41; $p = 0.002$) cells/μL (Fig. 1).

The distribution of MICs according to the molecular type for FLU are described in Table 2. Although we identified cryptococcal strains with decreased susceptibility to FLU, there were no significant differences between the incidence of FLU non-wild-type isolates and clinical presentation as well as CSF cytology, time to CSF sterilization, extraneural infection, previous diagnosis of CM or usage of FLU, and overall mortality. Moreover, no correlation was found between FLU susceptibility and molecular type. Furthermore, all *C. neoformans* isolates were of the *alpha* mating type. The correlations among clinical and laboratory data with the AMB–MICs were not assessed because all of the isolated yeasts had an MIC ≤ 1 μg/mL, which we classified as wild-type.

No association was found between the number of yeast cells and molecular type, time to CSF sterilization, ICP, current shunt, history of CM, or earlier use of FLU.

Cryptococcal Meningitis Prognostic Factors

There is great interest in developing interventions to reduce the mortality of patients with CM. Among the predictive factors associated with the worst prognosis and high mortality in these patients include alteration in mental status, *Cryptococcus* growth from sites other than the CSF, increased ICP, high CSF cryptococcal antigen titer, quantitative cryptococcal culture [37–39], seizures, and more than 14 days with symptoms [32••].

Beyond these associated factors, we found an important association between the initial CSF yeast cell counts as high as 400 cells/μ by direct microscopy and death. Thus, this metric could be used to discriminate patients with greater risk of death and call the attention of clinicians to identify patient prognosis and propose efficient strategies to reduce mortality. We believe that cell counts high values is associated with poor prognosis in HIV-infected patients with CM, and is a test that could be easily implemented in a laboratory setting. Thus, a study with a greater number of patients should be conducted, and the initial CSF yeast cell counts by direct microscopy should be compared with the quantitative CSF cultures.

In the present study, mortality was also associated with persistent positive CSF cultures during hospitalization. North American guidelines suggest that dual therapy should be maintained for a minimum of 2 weeks [15•]. However, in our clinical practice, we maintain treatment until patients present with a negative CSF culture after the second week of the induction phase treatment, which consequently extends the treatment duration to at least 4 weeks because it requires a 2-week incubation to obtain a culture result. Furthermore, we

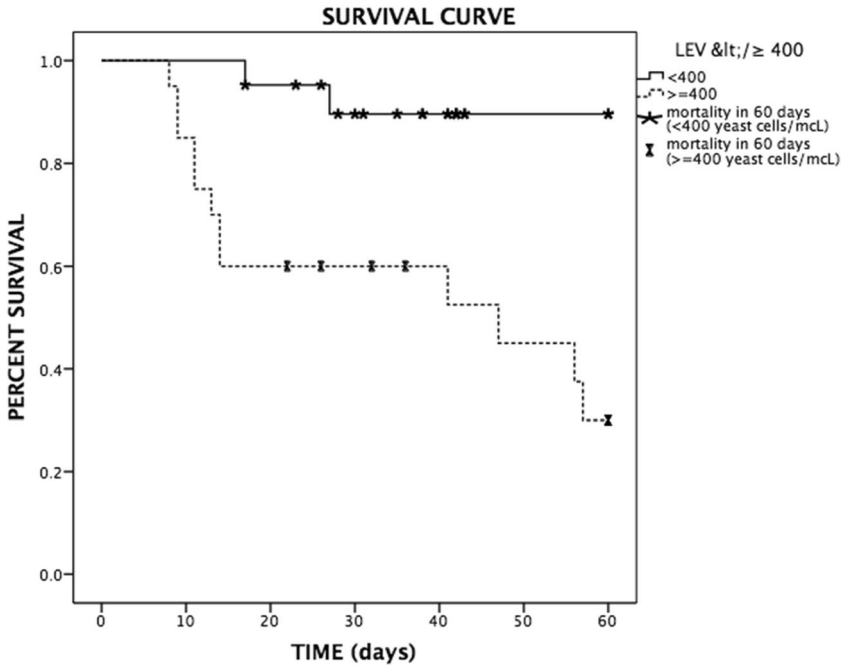

Fig. 1 Kaplan–Meier curve showing greater survival when initial cerebrospinal fluid yeast cell count was less than 400 cell/μL

Table 2 Susceptibility of 45 clinical isolates of *Cryptococcus neoformans* to fluconazole

Antifungal agent/molecular type (no. of isolates)	MIC (μg/mL)			No. of isolates with MIC (μg/mL)												No. of wild-type isolates, *n* (%)
	Range	50%	90%	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	
Fluconazole																	
VNI (39)	(0.12–64)	8	16	ND	ND	0	1	4	1	2	6	2	8	11	2	2	15/39 (38.4%)
VNII (6)	(2–64)	4	8	ND	ND	0	0	0	0	0	2	2	1	0	0	1	1/6 (16.6%)
Total (46)*	(0.12–64)	8	16	ND	ND	0	1	5	1	2	8	4	9	11	2	3	16/46 (34.7%)

MIC, minimum inhibition concentration; *ND*, not determined

*One strain could not be differentiated in VNI or VNII

observed that the mean time to CSF sterilization was 14 (range 6–62) days; however, our patients used the combined therapy for up to 33 days. An issue associated with this strategy was that we ended up treating patients for longer, which may lead to an increased risk of side effects related to AMB. Moreover, a recent study from our hospital observed that ≥ 10 yeasts/μ CSF between days 7 and 14 days was associated with a 98% CSF culture positivity. Thus, beyond a simple laboratory test, counting yeast could be a very useful clinical test to identify patients who have persistent positive cultures and who should be targeted with prolonged AMB induction therapy [32••].

In our study, although most patients presented with the cytological CSF characteristics associated with higher mortality, only a few patients presented with severe CNS symptoms such as altered mental status and seizures. The greater duration of symptoms in our cohort can be explained by the lack of severe symptoms but also by patient difficulties in accessing the healthcare system. Unfortunately, the quantification of the cryptococcal antigen titer, which is an important assay associated with poor prognosis [38, 39] was performed only in a qualitative manner in the present study. Regarding extraneural cryptococcosis, the data could be underestimated due to the lack of systematic investigation aiming to recover the etiological agent from other tissues.

We believe that controlling the ICP is a difficult management problem in CM [15•]. In our hospital, a systematically aggressive treatment for ICP was not performed because of the high number of lumbar punctures prior to insertion of the shunt and this practice could explain in part the high mortality among our patients.

Another plausible reason for our high mortality rate was related to the etiological agent; all *C. neoformans* were of the *alpha* mating type, which has been recognized as having a more pathogenic behavior due to higher melanin and urease production which enhance CNS invasion leading to a worse prognosis, compared with the *a* mating-type strains. Furthermore, in the *C. neoformans* var. *grubii* strains, there is no difference in the virulence of cells of opposite mating types; however, during co-infection, cells more easily cross the blood–brain barrier [40].

Only a few studies have been performed in Brazil to molecularly identify *C. neoformans* isolated from clinical samples, showing rates from 93 to 100% of the VNI. We found that almost 13% of our strains were VNII, which was higher than all other Brazilian studies; however, this fact was not associated with increased mortality [41–45].

There was no relationship between the in vitro susceptibilities of the infecting strain to antifungal agents and the clinical outcome, as previously reported [28•]. A study encompassing HIV-infected patients and 276 strains of *C. neoformans* from the CSF also showed no association between the MICs and mortality at day 14 and day 70 [46]. Until now, the unique clinical relevance of the MIC value is related to whether the treatment should be changed from FLU to voriconazole in cases of positive cultures after 4 weeks of treatment, whereby the strains have either an initial FLU–MIC ≥ 16 μg/mL or significantly increasing MIC values [15•]. Notably, among persistent CM cases, we observed 2 in 4 cases progressed to death, with one patient infected with a wild-type strain (FLU–MIC 4 μg/mL) and the other infected with a non-wild-type strain (FLU–MIC > 8 μg/mL). The remaining patients were infected by non-wild-type strains, stressing the poor clinical relevance of the MIC test. Despite the few studies that correlated the MIC values of FLU with the clinical aspects and outcomes in patients with CM, this metric should receive additional attention because it may be related to resistance genes against the main drug available for CM treatment in Latin America [29, 30•]. The importance of tracking the emergence of resistance is sustained by use of antifungal agents that continuously increase the selection pressure for resistance. Two multicenter studies were conducted to establish epidemiological cutoffs for FLU and AMB. The role of the ECV is to separate or distinguish wild-type from non-wild-type isolates. According to the authors, the rate of FLU non-wild-type MICs among *C. neoformans* was low, ranging from 1.7 to 9.5% [27••]. In fact, the AMB–MIC is of lower relevance in comparison to FLU–MIC, taking account homogenous and wild-type AMB–MIC distribution. Similar rates (1.5% to 2.8%) were described in a study of AMB non-wild-type isolates. In our study, although there were a small number of strains

tested, the frequency of FLU–MICs above the ECV (non-wild-type) was higher, particularly in *C. neoformans* VNI isolates, indicating the occurrence of isolates harboring resistance mechanisms [19, 47]. We observed totality of the AMB wild-type isolates infecting our patients, which ruled out any statistical analysis aiming to establish a clinical correlation. Most of studies found it difficult to discern the MIC impact due to the narrow AMB–MIC range observed with current testing methods [48]. Further investigation is needed to determine the relationship between the molecular mechanisms of FLU resistance and the proposed non-wild-type values.

The limitations of our study include the difficulty of evaluating the in vitro–in vivo correlation in a group of CM patients treated with combined therapy because synergy could occur even if the strain has an elevated MIC for one or more of the antifungal drugs [49, 50]. Moreover, the paucity of studies correlating the MICs with PK/PD in animal tests and clinical outcomes decreases the utility of the MIC test [51–53]. This fact should reassure us about the use of the MIC test and justify the need for new tools for determine the antifungal susceptibility profile with more evidence for clinical applications.

Moreover, the prognostic factors for mortality may be overstated because death may not be attributable to cryptococcosis itself because patients develop a great number of morbidities during hospitalization, including pneumonia, surgical complications, acute renal failure, blood stream infection, sepsis, and the presence of other opportunistic infections, all of which are highly common. Finally, the retrospective approach and small patient cohort may hinder or render impossible the analysis of the risk factors related to death in a multivariate analysis.

Conclusions

We concluded that the initial CSF yeast cell counts by direct microscopy is found to be an easy and affordable method, and high values (400 cells/μ) is associated with a poor prognosis in HIV-infected CM patients. Our findings corroborate with previous results obtained with various antifungal drugs that MIC do not predict early clinical outcomes in CM patients. The clinical role of MICs is uncertain, and there is no good evidence for their use in routine practice in the management of CM. We reinforce that CM is difficult to manage; that the aspects associated with greater mortality should be analyzed carefully; and that the conduits to control this disease, such as dual therapy, ICP control, and extended therapy for associated CNS lesions should be promptly performed.

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Compliance with Ethical Standards

Conflict of Interest Oscar José Chagas, Renata Buccheri, Márcia de Souza Carvalho Melhem, Walderez Szeszs, Marilena dos Anjos Martins, Lidiane de Oliveira, Rosa Marcusso and Daniel Wagner Santos declare no conflicts of interest relevant to this manuscript.

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- • Of importance
- •• Of major importance

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