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Evaluation of the in vitro activity of amphotericin B by time–kill curve methodology against large and small capsulate *C. neoformans* isolates

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Abstract

We have evaluated and compared the activity of amphotericin B (AMB) by time–kill curve methodology against 20 clinical *Cryptococcus neoformans* isolates in which capsule induction in vitro was performed. Overall, large capsulated isolates were more resistant to killing by AMB over time when compared with those small capsulate ones.

Keywords: Capsule; *Cryptococcus*; Time kill curves

1. Introduction

Cryptococcus neoformans is an encapsulated yeast with a worldwide distribution that causes disseminated disease, generally in immunocompromised individuals (Osuna et al., 2008; Rodero et al., 2000; Singh et al., 2008). *C. neoformans* is one of the most common causes of meningitis in HIV-positive patients. Despite the standard treatment with amphotericin B (AMB) and consolidation with fluconazole plus further prophylaxis, relapses often occur (Perfect et al., 2010).

In vivo, *C. neoformans* is encapsulated, with the capsule size varying depending on the infection site (Bose et al., 2003; Moyrand et al., 2002). The capsule serves to protect the fungal cells from phagocytosis and host immune responses and is an essential virulence factor (McFadden et al., 2007). In the cerebrospinal fluid (CSF) of infected individuals *C. neoformans* cells typically have capsules that are large enough to be easily identified. However, when the yeast is recovered and cultured under ordinary laboratory conditions, the capsule gradually becomes smaller. As a consequence, in vitro studies performed to evaluate the activity of antifungal agents against *C. neoformans*, such as determination of MIC, time–kill curves (TKC), or post-antifungal effect, have been carried out using small capsulate isolates (Ernst et al., 2000; Ghannoum et al., 1992; Rodriguez-Tudela et al., 2000).

To evaluate the antifungal activity of AMB under conditions that more closely resemble human disease, we performed TKC assays using *C. neoformans* isolates that had been induced to form capsules in vitro. These results were compared in both groups: those with capsule induction (large capsule strains) and those without capsule induction (small capsulate strains). Different conditions such as the use of bicarbonate, CO_2, and the media described below were used for capsule induction. We have noted that these test conditions had not influenced the activity of AMB since this drug was fungicidal for the control acapsular *C. neoformans* mutant ATCC 52817 both under and without those conditions.

2. Materials and methods

We tested 20 *C. neoformans* isolates isolated from the CSF of HIV-positive patients with cryptococcal meningitis. Two *C. neoformans* isolates were used as controls: ATCC

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90112 and ATCC 52817 acapsular mutant. All growth media and chemicals used were obtained from Sigma (Buenos Aires, Argentina).

Isolates were grown in Sabouraud glucose agar (SGA) and incubated for 48 h at 35 °C, resulting in small capsulate isolates. On the other hand, the same isolates were grown in SGA with 1.8 g/L of $NaHCO_3$ and incubated under 5% CO_2 atmospheric conditions to induce large capsule formation (Zaragoza et al., 2003).

A 10 μg/mL stock solution of AMB was prepared in 100% dimethylsulfoxide (DMSO) and stored at 70 °C until needed. A 1 μg/mL working solution was prepared by serial 10-fold dilutions in RPMI 1640 (RPMI) medium.

TKC assays were performed using a shaking incubator set at 350 rpm and 35 °C. All assays were performed in duplicate, and a control growth tube (in drug-free medium) was included in each assay. The isolates from the SGA in the conditions described above were grown in RPMI buffered with MOPS to pH 7.0. The initial inoculum was adjusted to 5×10^6 CFU/mL, by a hemocytometer counter, and then diluted 10-fold in RPMI containing 1 μg/mL of AMB. The small capsulate isolates were incubated in this condition, and to maintain the large capsule size in the other group, 22 nmol/L of $NaHCO_3$ was added to the RPMI and incubated under 5% CO_2 atmospheric conditions.

Colony counts were determined at 0, 6, 12, 24, 48, and 72 h. At each time point, aliquots were removed from each tube and serial 10-fold dilutions were made in sterile distilled water. Capsule size was checked over time in both groups, and the range size for large capsule isolates was 10 to 15 μm compared with 2 to 4 μm for the small capsulate ones.

Thirty-microliter aliquots were plated on SGA in Petri dishes, incubated at 35 °C, and colonies were counted after the times detailed above.

The assay end point was defined as a killing of ≥99.9% of the experimental cells compared to the drug-free controls or >2 log difference of the CFU between experimental cells and controls. CFU per milliliter counts were plotted against time for each of the isolates tested.

3. Results

Overall, large capsulated isolates were more resistant to killing by AMB over time. Consistent with previous reports (Pappalardo et al., 2009), 65% of small capsulate isolates in our study were killed by AMB after 6 h of incubation; however, 100% of large capsulated isolates survived at 12 h. As an example, the kill curves of *C. neoformans* strain ATCC 90112 differed based on encapsulation status, with <2 log difference in CFU compared with the drug-free control after 72 h of capsule induction compared to >2 log difference in CFU after 6–12 h without capsule induction (Table 1, Figs. 1 and 2).

By contrast, *C. neoformans* strain ATCC 52817 (acapsular mutant) was killed after 6 h under both conditions, indicating that the capsule influenced the activity of the drug (data not shown). The MICs of large and small isolates were within the range of 0.25 to 1 μg/mL (data not shown).

Table 1
Comparison of AMB action over time between encapsulated and capsulate-deficient isolates

C. neoformans (n = 20)	Percentage (%) of strains that survived over time				
	6 h	12 h	24 h	48 h	72 h
Small capsulate isolates	35	15	15	0	0
Large capsulated isolates	100	100	95	80	75

4. Discussion

Current methodologies to evaluate the MIC of AMB fail to discriminate between susceptible and resistant isolates of *C. neoformans* (CLSI, 2002; Pfaller et al., 2004). The MIC values reported for AMB generally fall within the range of 0.5 to 2 μg/mL (Ernst et al., 2000). With the large capsulated isolates of *C. neoformans*, we observed a similarly wide range (data not shown).

TKC might be a better predictor of clinical outcome than standard in vitro susceptibility testing, which can underestimate the resistance of *C. neoformans* to AMB in vivo (Pappalardo et al., 2009; Rodero et al., 2000).

In one study, it was described that capsules could reduce the activity of AMB, which is in agreement with our findings (Zaragoza et al., 2008). However, to our knowledge, this is the latest report to describe AMB activity against large capsulated *C. neoformans* in TKC assays. Measurement of antifungal activity over time is a more dynamic parameter than MIC determination based on in vitro susceptibility assays.

Our results suggest that large capsulated isolates might be more tolerant of AMB action, but translating our findings to the clinic is not straightforward.

Interesting to mention are two clinical trials that measure *C. neoformans* colonies count over time from CSF obtained

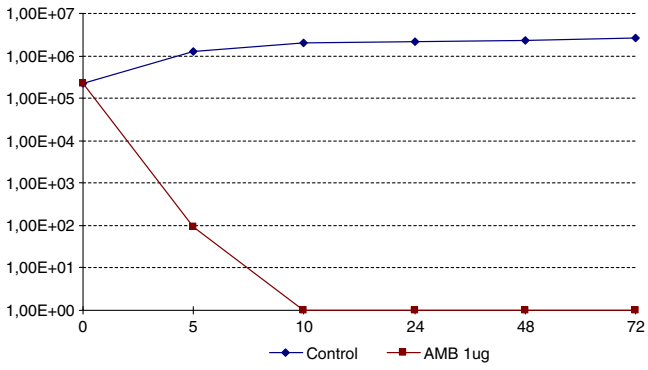

Fig. 1. Time–kill curve of *C. neoformans* ATCC 90112 without capsule induction.

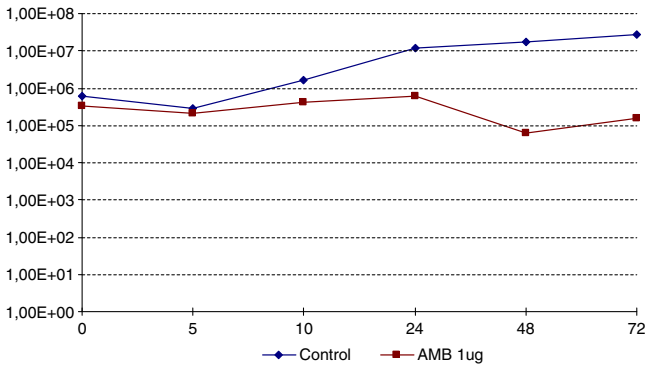

Fig. 2. Time–kill curve of *C. neoformans* ATCC 90112 with capsule induction.

from patients suffering from cryptococcosis, during an in vivo TKC examination. In these studies, the distinction between large and small capsulate strains was not mentioned. One study is a clinical trial performed in Africa that described, from serial CSF functions, sterilization of the liquor, as measured by the rate of reduction in CSF cryptococcal colony-forming units when fluconazole was combined with flucytosine (Nussbaum et al., 2010). The other study, performed in Thailand using the same methodology, has shown more fungicidal activity of AMB in combination with other antifungal agents compared to when it was administrated alone (Brouwer et al., 2004).

Thus, it might be useful to determine the TKC pattern of clinical isolates under conditions of capsule induction, with the goal of changing antifungal therapy if tolerance is observed. Besides, these tools, the in vitro–in vivo TKC examination together, could help to improve treatment regimens.

However, more investigation is needed in order to determine how these findings might correlate with clinical outcome and whether TKC patterns measured under capsule induction could predict clinical failure.

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