Journal of Medical Microbiology (2012), **61**, 384–388 **DOI** 10.1099/jmm.0.036152-0

Influence of capsule size on the *in vitro* activity of antifungal agents against clinical *Cryptococcus neoformans* var. *grubii* strains

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Received 13 July 2011
Accepted 8 November 2011

Cryptococcosis causes disseminated disease in AIDS patients. In contrast to what occurs in laboratory conditions, a large capsule is produced by *Cryptococcus neoformans in vivo* during infection. The aim of this study was to compare the *in vitro* activity of different antifungal agents against 34 clinical isolates of *C. neoformans* var. *grubii* without or with capsule induction (CLSI, CLSI-C, respectively), following the CLSI M27A3 document. Capsule induction was obtained by addition of $NaHCO_3$ and incubation with CO_2. The geometric means of the MICs, in μg ml^{-1}, for CLSI and CLSI-C cultures, respectively, were 1.9 and 9.8 for fluconazole; 0.04 and 0.08 for itraconazole; 0.04 and 0.05 for voriconazole; 0.16 and 0.38 for amphotericin B; and 1.6 and 5.6 for 5-flucytosine. Thus fluconazole showed the highest MICs after capsule induction. Determination of antifungal activity after capsule induction may be clinically relevant and could be used to evaluate the correlation between *in vitro* results and clinical outcome.

INTRODUCTION

Cryptococcosis with involvement of the central nervous system is commonly encountered among HIV-infected patients, and approximately 90 % of AIDS patients infected with *Cryptococcus neoformans* develop meningitis (Nguyen *et al.*, 2010; Osuna *et al.*, 2008). *C. neoformans* has several virulence factors. Presence of a capsule is the most prominent of these, and it has been shown that capsular polysaccharides have deleterious effects on the immune system (Bahn *et al.*, 2005; Granger *et al.*, 1985).

The size of the capsule of *C. neoformans* is variable, depending on the environmental conditions. Thin capsules are observed in standard fungal culture media *in vitro* under laboratory conditions. However, during *in vivo* infection, mainly in cerebrospinal fluid (CSF), the yeasts have large capsules (Frases *et al.*, 2009; Rivera *et al.*, 1998).

Several methodologies to evaluate the antifungal activity of *C. neoformans* have been proposed by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS), in Document M27A3 (CLSI, 2008), but all these procedures have to be performed under laboratory conditions, in which *C. neoformans* exhibits a small capsule size.

Some studies have evaluated the antiphagocytic properties of *C. neoformans* after capsule induction by CO_2 and sodium bicarbonate (Bahn *et al.*, 2005; Granger *et al.*, 1985; Zaragoza *et al.*, 2003). However, to our knowledge the antifungal activity of different drugs has never been evaluated by the CLSI methodology after capsule induction. Thus, the aim of this work was to study the *in vitro* activity of different antifungal agents against *C. neoformans* var. *grubii* in which *in vitro* capsule growth had been induced to mimic the *in vivo* situation and to compare these results with those obtained from the same strains without capsule induction.

Abbreviations: AMB, amphotericin B; CSF, cerebrospinal fluid; 5FC, flucytosine; FCZ, fluconazole; Gmean, geometric mean; ITZ, itraconazole; VCZ, voriconazole.

METHODS

Antifungal agents. The drugs tested were amphotericin B (AMB) (Bristol Myers Squibb); fluconazole (FCZ) and voriconazole (VCZ) (Pfizer); itraconazole (ITZ) (Janssen Farmacéutica) and flucytosine (5FC) (Sigma Aldrich).

Organisms and growth conditions. Thirty-four strains of *Cryptococcus neoformans* isolated from CSF of HIV-positive patients were evaluated. *Candida krusei* ATCC 6258, *Candida parapsilosis*

ATCC 22019, *Cryptococcus neoformans* ATCC 90112 and *C. neoformans* acap70 ATCC 52817 (acapsular mutant) were used as quality-control strains. All strains were grown on Sabouraud glucose agar (SGA) at 35 °C for 48 h before the susceptibility tests.

Molecular identification. DNA was isolated from the 34 strains following a standard protocol (Liu *et al.*, 2000).

To determine the varietal status of the clinical isolates, the *SOD1* allele was amplified by PCR using primers specific for *C. neoformans* var. *grubii* and *Cryptococcus gattii* with the isolated DNA as template. Primers JOHE7777/7775 for *C. neoformans* var. *grubii* and JOHE7773/7775 for *C. gattii* were used (D'Souza *et al.*, 2004). Positive controls (*C. neoformans* var. *grubii* CBS 8710 and *C. gattii* WM178) were included.

To determine the serotype and mating type of the clinical isolates, PCR was performed to amplify the mating type α or a and serotype A or D allele of the *STE20* locus using specific primers JOHE7270/7272 (aA), JOHE7273/7275 (aD), JOHE7264/7265 (αA) and JOHE7267/7268 (αD) (Barreto de Oliveira *et al.*, 2004). Reference strains CBS 9172 (aA), CBS 8710 (αA), CBS 10511(aD) and CBS 10513 (αD) were included. The mating type α and a primers yielded a 1.2 kb and an 870 bp product, respectively.

Capsule induction. To induce capsule formation the yeast cells were inoculated on SGA with the addition of 22 mM $NaHCO_3$ and incubated in the presence of 5 % CO_2 at 30 °C for 48 h.

Measurement of capsule. To visualize the size of the capsule a drop of Indian ink was added to a cell suspension. To calculate the size of the capsule the diameter of the cell plus capsule and the cell alone were measured using an optical microscope with a magnification of ×400 using a calibrated micrometer eyepiece. Capsule size was obtained by the difference between the size of the cell plus capsule, and the size of the yeast cell alone. Thirty measurements were made for each determination, and the mean was calculated.

Antifungal susceptibility testing. The MICs were determined following the CLSI M27A3 guidelines (CLSI, 2008) in two conditions: (a) without capsule induction (CLSI) and (b) with capsule induction (CLSI-C). For (a), the isolates were incubated for 48 h on SGA before the tests. The medium used for the tests was RPMI 1640 without sodium bicarbonate, with L-glutamine (Sigma Aldrich), buffered with MOPS (Sigma Aldrich) at pH 7. For (b), after capsule induction as described above, the isolates were grown in RPMI 1640 medium containing 22 mM $NaHCO_3$, with L-glutamine, buffered with MOPS at pH 7, and the incubation was performed in the presence of 5 % CO_2 to allow capsule induction. The inoculum was adjusted by haemocytometer measurements. The starting inoculum was $0.5–2.5 \times 10^6$ c.f.u. ml^{-1}, and the final inoculum was $0.5–2.5 \times 10^3$ c.f.u. ml^{-1}, for both (a) and (b).

The inoculum size was checked by plating serial dilutions on SGA and c.f.u. were determined after incubation at 35 °C for up to 72 h.

AMB, ITZ and VCZ were diluted in DMSO at 3200 μg ml^{-1}, whereas FCZ and 5FC were diluted in water at 12 800 μg ml^{-1}. Serial dilutions of the drugs were made following the CLSI guidelines in order to obtain final concentration ranges of 16–0.03 μg ml^{-1} for AMB; 8–0.007 μg ml^{-1} for ITZ and VCZ, 64–0.06 for FCZ and 32–0.03 for 5FC. The tests were performed in 96-well flat-bottomed microtitre plates (Becton Dickinson). Yeast suspensions were diluted 1 : 10 and 1 : 100 in water and in liquid RPMI 1640 medium (see above). The plates were incubated at 35 °C for 72 h, then scanned at 450 nm with a Labsystems Multiskan RC microplate spectrophotometer (Labsystems Multiskan).

The MIC end point for AMB was defined as the lowest concentration of the drug that showed 100 % inhibition compared with the growth control; for the other drugs it was defined as the lowest concentration that showed 50 % of inhibition compared with the control. All tests were performed in duplicate on two different days.

Statistical analysis. The high and the low off-scale MICs were included in the analysis. High off-scale MICs were converted to the next higher concentration and the low off-scale MICs were converted to the next lower concentration. The differences between MICs obtained by the different methods were analysed by Mann–Whitney *U* test. The significance level of 0.05 was chosen.

RESULTS

In vitro capsule induction

Capsule size was measured after incubation on SGA with or without induction by $NaHCO_3$ in the presence of 5 % CO_2. For the *C. neoformans* acapsular mutant ATCC 52817, no modification was observed under these conditions. For the *C. neoformans* clinical strains and the reference strain ATCC 90112, capsule size was significantly increased from 1–3 μm to 10–15 μm after induction.

The geometric mean (Gmean) in μm, for capsule size without and with *in vitro* induction for acapsular *C. neoformans* ATCC 52817 was 1 for both conditions; for the clinical isolates of *C. neoformans* var. *grubii* (n=34) it was 2.6 and 12.1, respectively.

The MICs for the control strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were within the expected range proposed by the CLSI M27A3 document.

Molecular analysis of clinical isolates

The PCR with *SOD1-C. gattii*-specific primers yielded products with the reference *C. gattii* strains, but no product with the clinical isolates was detected, indicating that none of these strains were *C. gattii*. The *C. neoformans* var. *grubii*-specific primers gave PCR products with all the samples tested, indicating that all of them were *C. neoformans* var. *grubii*. PCR amplification using primers specific for the *STE20* gene showed that all the isolates were of serotype A. For the mating type test, the αA PCR gave positive results with all the strains except one, which was an aA isolate.

Antifungal susceptibility testing

In general, higher MIC values were observed for encapsulated strains. FCZ was the least active drug: the Gmean MICs (μg ml^{-1}) for CLSI and CLSI-C were 1.9 and 9.8 (P<0.05). Fourteen strains with small capsules for which the MIC values were low showed an increase in MIC of more than two dilution steps when tested with a large capsule (Fig. 1a). 5FC and AMB also showed a significant difference in MIC between the two methodologies, the respective Gmean values being 1.6 and 5.6 μg ml^{-1} for 5FC (Fig. 1e), and 0.16 and 0.38 μg ml^{-1} for AMB (Fig. 1d), for CLSI and CLSI-C, respectively (P<0.05). However, for 5FC only in eight strains was a difference of more than two dilution steps observed. For the other drugs no statistically significant difference was

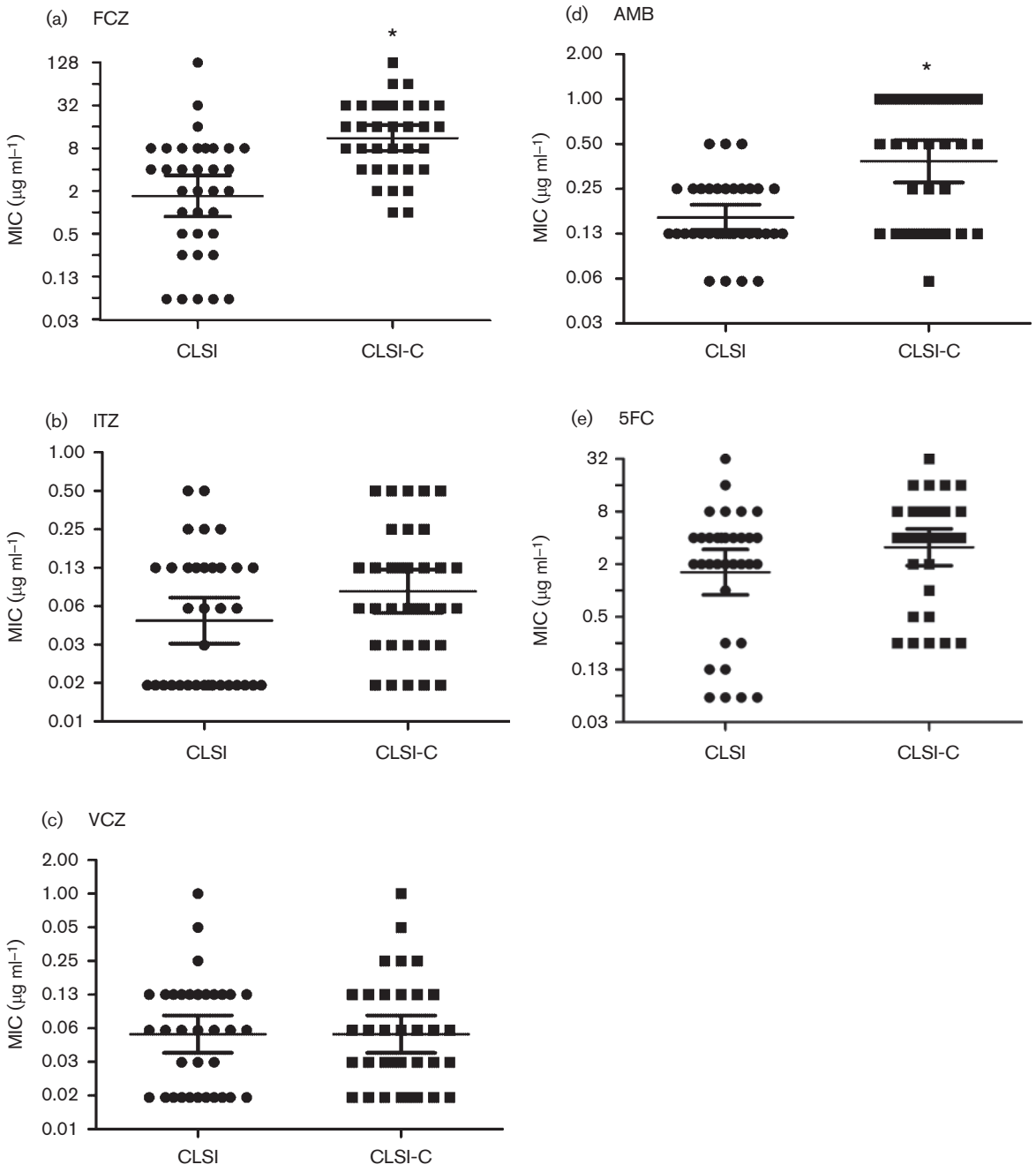

Fig. 1. Comparison of the MICs (μg ml^{-1}) between CLSI and CLSI-C methodology for the antifungal agents tested. *, $P<0.05$.

observed ($P>0.05$): the respective Gmean MICs in μg ml^{-1} determined by CLSI and CLSI-C, respectively, were 0.04 and 0.08 for ITZ (Fig. 1b), and 0.04 and 0.05 for VCZ (Fig. 1c). All data are summarized in Table 1.

DISCUSSION

Cryptococcus neoformans has a large polysaccharide capsule that is necessary for virulence and is the target of a protective antibody response (Frases *et al.*, 2009; Zaragoza *et al.*, 2009). Variation of capsule size between different cryptococcal isolates during the course of human infection has been reported (Cherniak *et al.*, 1995), and capsule size may also vary depending on the site of infection (Rivera *et al.*, 1998). The mechanisms responsible for changes in the thickness of the capsule could be related to different proteins involved in its biosynthesis (Janbon *et al.*, 2001). The gas carbon dioxide (CO_2) plays a critical role in microbial and mammalian respiration and it is known to

Table 1. Gmean MICs, MIC_{50}, MIC_{90} and MIC range of the drugs tested against *C. neoformans* var. *grubii*

All values are in μg ml^{-1}.

Drug	(a) CLSI				(b) CLSI-C			
	Gmean	MIC_{50}	MIC_{90}	Range	Gmean	MIC_{50}	MIC_{90}	Range
AMB	0.16	0.125	0.25	0.06–0.5	0.38	0.5	1	0.06–1
ITZ	0.04	0.06	0.25	0.015–0.5	0.08	0.06	0.5	0.015–0.5
VCZ	0.04	0.06	0.125	0.015–0.5	0.05	0.06	0.25	0.015–1
FCZ	1.9	4	8	0.06–128	9.8	16	32	0.5–128
5FC	1.6	2	8	0.031–32	5.6	8	16	2–32

induce production of polysaccharide capsule in pathogenic bacteria and fungi (Bahn *et al.*, 2005). However, the mechanism by which this occurs has not yet been clarified. In human infection, larger *C. neoformans* capsule sizes were especially observed in CSF (Bose *et al.*, 2003).

The role of the capsule in the antifungal susceptibility of *C. neoformans* is poorly known. Therefore, we studied antifungal activity against isolates of *C. neoformans* var. *grubii*, identified by classical and molecular techniques, in which capsule production was induced *in vitro* by incubation with bicarbonate and CO_2. The results were compared with those obtained for the same strains by cultivation under standard laboratory conditions. There is no standard definition of 'small' and 'large' capsules. In one study of proven pulmonary cryptococcosis, *C. neoformans* cells with a capsule size $\leqslant$1 μm were called capsule deficient, whereas those with a capsule size $>$1 μm were defined as having a large capsule (Torres *et al.*, 2005). In the present study, we defined small capsulated strains as those with capsule sizes 1–3 μm and large capsulated strains as those with capsule sizes $>$3 μm.

All the strains tested when incubated in the CLSI standard conditions showed a capsule size of 1–2 μm, whereas with the addition of $NaHCO_3$ and in the presence of CO_2, the size increased up to 15 μm, depending on the strain. In contrast, no induction was observed for the control strain, the acapsulate mutant *C. neoformans* ATCC 52817.

The presence of bicarbonate and CO_2 did not affect the growth of the *Candida* control strains or the *C. neoformans* isolates, which is in agreement with another study in which hypoxic conditions and presence of CO_2 did not influence growth of *Candida* and *Aspergillus* (Warn *et al.*, 2004). On the other hand, the antifungal MICs for the *Candida* control strains were similar in the presence or absence of bicarbonate and CO_2, indicating they did not influence the MIC determination.

The MICs observed in this study differed significantly among the drugs and the methodologies. Capsule size is altered by VCZ (van Duin *et al.*, 2004) and this effect could be related to the fact that no differences were observed in the MIC of this drug between small and large capsulated strains.

FCZ was the least active drug in the presence of a large capsule. The reason why capsulated strains are less susceptible to this azole cannot be explained by the results of the present study. It is possible that capsule components reduce the ability of this drug to attach to the target, resulting in the lack of action observed. However, for the other azoles this effect was not observed. It may be necessary to study this phenomenon at molecular level in order to elucidate if the mechanism of action in encapsulated *C. neoformans* strains varies among the azoles. The finding that FCZ is less active is remarkable, since this drug is used in treatment of cryptoccocal meningitis after AMB therapy.

For 5FC, significant differences in antifungal activity were observed between large and small encapsulated strains, but the difference was no more than two dilution steps for most of the strains. The MIC values observed in this work are similar to those reported by other authors that evaluated the activity of 5FC against *C. neoformans* without capsule induction (Chowdhary *et al.*, 2011; Dannaoui *et al.*, 2006). It seems that the polysaccharide capsule may affect the activity of 5FC differently from the other drugs; this could be related to the small size of this drug and its mechanism of action.

AMB was significantly less active for large capsulated strains. In one study it was demonstrated that AMB may affect the capsule size in a murine model (Zaragoza *et al.*, 2005). In our *in vitro* study, however, it seems that capsule could affect the activity of AMB. These are interesting observations, despite the fact that the two studies differed in the methodology used. Since when present in the CSF *C. neoformans* is usually seen with a large capsule, our observation of the effect of encapsulation on the activity of this polyene could be one of the reasons why in several clinical trials of AMB alone, it showed little ability to remove the fungus from the CSF in patients with disseminated cryptococcosis with CNS involvement (Perfect *et al.*, 2010).

In conclusion, this work has demonstrated that the presence of a polysaccharide capsule can influence the susceptibility of *C. neoformans* var. *grubii* to some antifungal agents. *In vitro* susceptibility tests of *C. neoformans* are routinely performed using strains with a thin capsule,

which is how *C. neoformans* appears outside the human body. Because during human infection, yeast cells with large capsules are generally present, performing MIC determinations after capsule induction could be a promising tool. It will be necessary to evaluate the optimal conditions to analyse the *in vitro* susceptibility of *C. neoformans* in order to find better approaches to correlate the results of *in vitro* tests with the clinical outcome. Thus, further studies are needed in order to elucidate the usefulness of these findings.

ACKNOWLEDGEMENTS

The authors acknowledge Eric Dannaoui for helpful discussions on the paper, Ferry Hagen for providing the *C. neoformans* variety *grubii* and *C. gattii* reference strains and Laura Rodero for providing several of the tested strains.

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