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Determination of the minimum inhibitory concentration of *Cryptococcus neoformans* and *Cryptococcus gattii* against fluconazole by flow cytometry

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Abstract

Recent studies have used flow cytometry (FCM) as an important alternative method to determine the antifungal susceptibility of yeasts compared to the broth microdilution Clinical and Laboratory Standards Institute (CLSI) reference procedure. We present a comparative study of the broth microdilution method and flow cytometry to assess the *in vitro* antifungal susceptibility of *Cryptococcus neoformans* ($n = 16$) and *C. gattii* ($n = 24$) to fluconazole. The minimum inhibitory concentration (MIC) assays by flow cytometry were defined as the lowest drug concentration that showed ~50% of the count of acridine orange negative cells compared to that of the growth control. Categorical classification showed all *C. neoformans* isolates were susceptible to fluconazole. Three isolates of *C. gattii* were susceptible dose-dependent and the remaining 21 isolates were classified as susceptible. MICs comparison of both methodologies demonstrated 100% categorical agreement of the results obtained for *C. neoformans* and *C. gattii*. The MICs obtained with the CLSI-approved method and flow cytometry were compared by the Spearman correlation test and a significant Pv = 0.001. The flow cytometric method has the advantage of analyzing a large and constant number of cells in less time, i.e., 9 h incubation for fluconazole using acridine orange versus 72 h for broth microdilution method. In conclusion, the two methods were comparable and flow cytometry method can expedite and improve the results of *in vitro* susceptibility tests of *C. neoformans* and

C. gattii against fluconazole and also allows comparative studies *in vitro*/*in vivo* more rapidly, which along with clinical data, could assist in selecting the most appropriate treatment choice.

Key words: flow cytometry, antifungal susceptibility, *Cryptococcus*.

Introduction

Cryptococcus neoformans is a pathogenic fungus, the cosmopolitan agent of fungal meningoencephalitis in immune-compromised patients [1] including a high number of occurrences in acquired immunodeficiency syndrome (AIDS)-related deaths in sub-Saharan Africa [2], as well as being recognized as an important infectious agent throughout the world [3]. The mortality rate in infections caused by this fungus is still high, especially when diagnosis is delayed and the access to highly active antiretroviral therapy (HAART) in AIDS patients is limited [4].

Cryptococcus gattii, a close sibling species of *C. neoformans*, is endemic in tropical and subtropical climates [5], causing meningoencephalitis with high death rates in hosts with normal immunity, especially in the emerging countries [6–9]. In the humid Brazilian Amazon (North) and in the semi-arid Northeast regions, *C. gattii* is the causative agent of meningitis in children and adolescents with high mortality in these groups, despite the use of colloidal amphotericin B (AMB) [10,11]. It has been speculated that *C. gattii* is more virulent and less susceptible to some antifungal drugs than *C. neoformans* based on *in vitro* investigations [12].

High doses of AMB associated with 5-fluorocytosine are indicated for induction therapy for cryptococcosis, followed by fluconazole (FCZ) in the consolidation phase [3]. FCZ is also indicated for the primary treatment of localized infections without evidence of dissemination [13,14]. Due to the increased significance of fungal infections and the introduction of new antifungal drugs, the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) in the USA has developed (since 1985) protocols for *in vitro* susceptibility testing to aid physicians in their therapeutic choices for treating cryptococcosis. The standardization and reproductibility of broth microdilution methodology for *in vitro* susceptibility testing for yeasts, current CLSI M27-A3 [15,16], allows greater comparability of results among different laboratories. However, this method is considered laborious and time-consuming because it takes 24/48 h for incubation of *Candida* spp. and 72 h for *Cryptococcus* spp. and training of personnel, mainly to appropriately obtain the data from the testing. This encouraged the search for new methods to reduce incubation time and automate the collection of results. Recent studies have combined the use flow cytometry (FCM) with the conventional method CLSI M27-A3/M27-S3 and found it to be an important alternative procedure to establish the *in vitro* antifungal susceptibility of yeasts. FCM is a powerful technique for the diagnosis of hematological diseases and is widely used in clinical laboratories [17,18]. This technique has shown great potential in the classification of tumors of hematopoietic and lymphoid tissue using morphological phenotypic and genotypic parameters, as recommended by Bethesda International Consensus in 2006 to study the Linfocitosis in FCM [18]. In addition, FCM combined with specific fluorochromes, like propidium iodide (PI), FUN-1 [2-choro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1 phenylquinolinium iodide] and acredine orange (AO), has been used by many authors to determine the *in vitro* susceptibility of yeasts, especially *Candida* spp. The MIC values generated through the use of FCM are based on the analysis of large numbers of individual yeast cells which does not require protracted incubation periods [19–22]. However, there have been very few studies which have examined the application of this method in assessing the antifungal susceptibility of *C. neoformans* and *C. gattii* [22].

Therefore, this work aims to optimize the *in vitro* antifungal susceptibility assay to obtain results in a shorter period of time for *C. neoformans* and *C. gattii* against fluconazole, the most common antifungal drug used in the treatment of cryptococcosis. For this we performed a comparison of MICs obtained by flow cytometry and the microdilution method (CLSI-M27-A3/M27-S3).

Material and methods

Strains

A total of 16 isolates of *C. neoformans* and 24 of *C. gattii* strains stored at the Culture Collection of Pathogenic Fungi in Evandro Chagas Clinical Research Institute at Oswaldo Cruz Foundation (IPEC/FIOCRUZ) at −70°C in 15% glycerol were employed in the studies. Purity, viability and genus identity of the strains were confirmed through the use of Niger seed agar medium (NSA) and biochemical tests (Vitek YBC, bioMérieux, Inc., Durham, NC, USA). The species were identified with CGB (canavanine-glycine bromothymol blue) medium test [23]. *Candida parapsilosis* INCQS 40038 (ATCC 22019) and *Candida krusei* INCQS

40147 (ATCC 6258) were included in the study as quality controls.

Antifungal agents

Fluconazole was obtained in powder form (Sigma-Aldrich Inc., St Louis, MO, USA). Stock solutions were prepared in dimethylsulfoxide-DMSO P.A. 99.70% (Vetec Fine Chemicals Ltd, Duque de Caxias, Brazil) at 5120 μg/ml and maintained at −70°C for up to 12 months [16].

Antifungal susceptibility testing CLSI

The microdilution tests were performed following the CLSI M27-A3 and M-27S3 broth microdilution guidelines [15,16]. Fluconazole dilutions ranging from 0.12–64 μg/ml were prepared in flat-bottomed 96-well microplates using RPMI-1640 broth medium with L-glutamine without bicarbonate (Gibco™, New York, USA) buffered at pH 7.0 with (3-[N-Morpholino]- propanesulfonic acid) buffer >99% (Vetec Fine Chemicals Ltd, Duque de Caxias, Brazil), and supplemented with D-(+)-Glucose 2% (Sigma-Aldrich Inc., St Louis, MO, USA), modification accepted by the CLSI document [16,24]. The inocula were obtained from 48-h-old cultures of each test strain grown on drug-free Sabouraud dextrose Agar at 35°C. A pilot study showed that the growth of *Cryptococcus* cells was far better using 0.45% saline solution rather than 0.85% in a short incubation time (2–12 h), so that FCM results could be reproducible. Therefore, the suspensions were prepared with 0.45% sterile saline and adjusted using Bio-Mérieux Densichek instrument to match the turbidity of a 0.5 McFarland standard. The final concentration of the inoculum in the microplates was 0.5×10^3 to 2.5×10^3 CFU ml^{-1} using RPMI1640 broth medium as described above.

Antifungal susceptibility testing by flow cytometry (FCM)

The minimum inhibitory concentration (MIC) of fluconazole was determined by a flow cytometric approach using acridine orange as fluorochrome (AO) (Sigma-Aldrich Inc., St Louis, MO, USA).

Fluconazole solutions were prepared following the broth microdilution guidelines [15] using RPMI 1640 media as described above. In order to reduce the dilution range of fluconazole we used final concentrations of from 1–64 μg/ml. Three hundred microliters were distributed in capped sterile tubes specific to flow cytometry and kept at −70°C up to 6 months or until used in the study.

To prepare the inoculums suspensions, strains were grown in Petri dishes containing drug-free Sabouraud dextrose agar (Difco Laboratories, Sparks, MD, USA) at 35°C for 48 h. Five to 10 colonies of approximately 1 mm in diameter each were suspended in sterile 0.45% saline, the turbidity was adjusted using Bio-Mérieux Densichek instrument at a wevelength of 530 nm to 1 McFarland standard (approximatly 300×10^6 CFU ml^{-1}). Portions of this inoulum suspension (300 μl) were distributed in tubes containing 300 μl of one of the drug dilutions (2–128 μg/ml).

To evaluated the action of AO at a final concentration of 11 mg/l as described in tests involving *Candida* spp. [25], cells of *C. neoformans* and *C. krusei* were analyzed with fluorescence microscopy (Olympus B × 40: 1). Cells suspension in sterile 0.45% saline matching turbidity of 0.5 McFarland were incubated for 30 min with AO in the dark and were defined as negative control or AO negative (viable cells) if green cells were observed. In contrast, cells treated with formaldehyde at a final concentration 1% incubated for 1 h prior to addition of the AO for 30 min were defined as positive control or AO positive (non-viable cells) when orange-red cells were found (data not shown). To determine the MIC obtained with flow cytometry for *C. neofomans* and *C. gattii*, we performed a pilot study using AO at 11 mg/l as an indicator of viability. The MIC of FCZ was defined as the lowest concentration that showed ∼50% of viable cells, corresponding to the left quadrant of dot plot (Fig. 1).

Inoculum dilution acquisition was performed in Beckman Coulter XL-MCL flow cytometer and studies were conducted with a flow cytometric protocol in which size forward scatter (FSC) and granularity side scatter (SSC) at 675 nm fluorescence emission (FL4) 620V were measured. Data analysis was performed by a dual-parameter dot plot – FSC vs. AO fluorescence intensity, which was divided into two quadrants, i.e., left comprehending AO negative cells (viable cells), and right comprehending AO positive cells (non-viable cells). A gate was created (for *Cryptococcus* and *Candida*) in order to exclude debris and define the cell populations of interest.

In order to define the best incubation time for an appropriate reading in flow cytometry, we tested six representative strains of *C. neoformans* and *C. gattii*, and the quality control strains of *Candida krusei* and *C. parapsilosis*. The strains were incubated with continuous shaking at 200 rpm using Tecnal TE-420 incubator for 4, 8, 9, 12, 19 and 24 h at 35°C. After 30-min incubation with AO at 200 rpm, 10,000 cells were acquired in triplicate in flow cytometry. The lowest concentration which showed ∼50% (MIC2) viable cells after incubation of the inoculum cells for 4, 8, 9, 12, 19 and 24 h were compared to the concentrations range of the quality control strains (*C. krusei* and *C. parapsilosis*) from the CLSI guidelines [15].

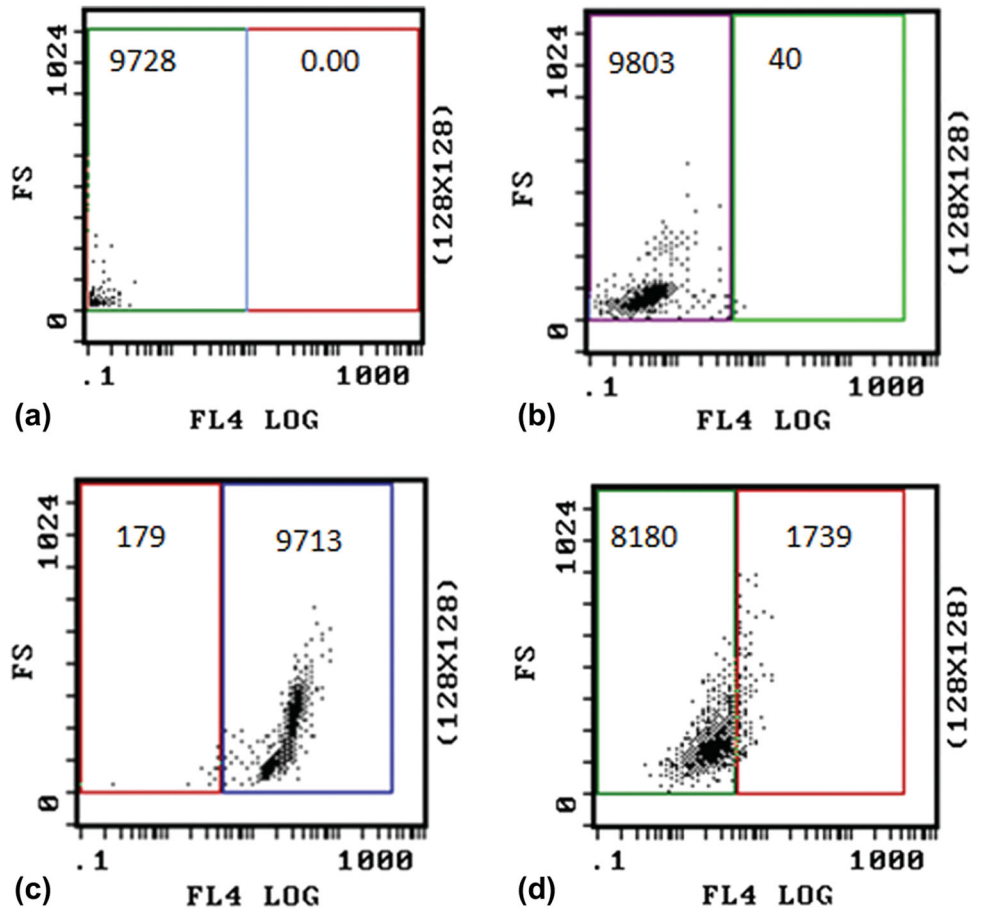

Figure 1. Dot plot of the controls for *Cryptococcus neoformans*: (a) autofluorescence; (b) *C. neoformans* stained with acridine orange (AO) for 30 min; (c) *C. neoformans* dead cells, after 1-h incubation with 1% formaldehyde and stained with AO for 30 min; (d) Live cells, incubation at 200 rpm for 9 h and stained with AO for 30 min.

Once the incubation time was defined, MIC determinations of each of the 40 strains were carried out at two different stages to check reproducibility. Controls AO positive and AO negative were prepared for each strain according to the same test conditions described above (Fig. 1).

Analysis of results and statistics for microdilution (CLSI)

After 72-h incubation for *C. neoformans* and *C. gattii*, and 24–48 h for *C. krusei* and *C. parapsilosis* at 35°C, MICs were determined spectrophotometrically with microplate reader EPOCH. For the spectrophotometrical reading, Microsoft Excel software was used to determine the MIC2 (~50% inhibition growth) for fluconazole.

Analysis of results and statistics for flow cytometry

The MIC results obtained from flow cytometry were analyzed with the Statistical Package for Social Sciences (SPSS) version 16.0. The Spearman correlation coefficient was used to compare the MICs obtained by CLSI and FCM. The categorical classification for *Candida* sp. was used to interpretate *Cryptococcus* spp. MICs obtained by CLSI and FCM as the breakpoints for *Cryptococcus* species are not stablished [26,27].

Results

The pilot study defined 9 h as the shorter time incubation period in which to obtain comparable MICs with FCM and CLSI methodologies, which adopts MIC limits: of 0.5–4.0 μg/ml for *C. parapsilosis* and 16–64 μg/ml for *C. krusei* in 24 h (Fig. 2). MIC ranges of the two quality control strains were within the CLSI established limits.

The MICs for fluconazole against *C. neoformans* and *C. gattii* obtained by CLSI and flow cytometry methods are shown in Table 1. The final MICs obtained with FCM and CLSI procedure ranged from <1–32 μg/ml (Table 1). The MIC_{50} and MIC_{90} were 2 and 16 μg/ml for FCM and 1 and 4 μg/ml for CLSI. Categorical classification showed

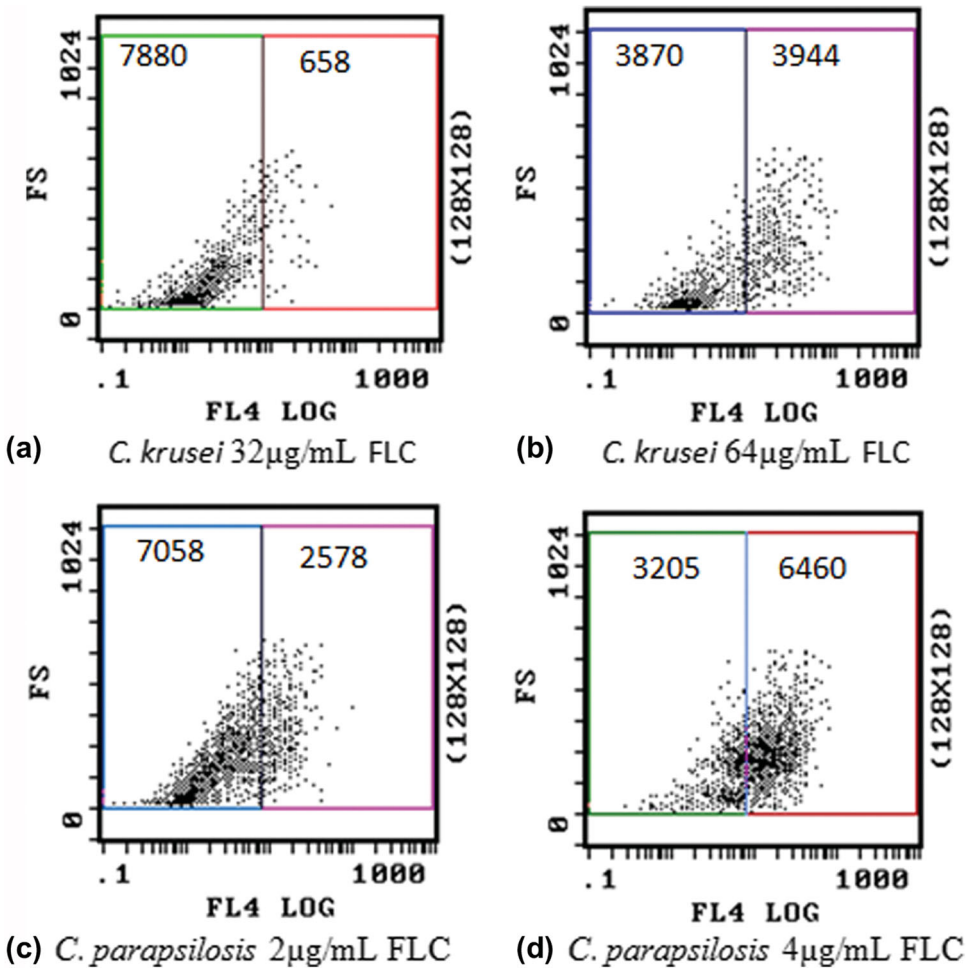

Figure 2. Dot plot of the corresponding MIC and the previous dilution obtained from the quality control strains, *Candida krusei* INCQS 40147 (ATCC 6258) and *Candida parapsilosis* INCQS 40038 (ATCC 22019): (a) previous dilution of MIC *C. krusei*; (b) MIC of *C.krusei*; (c) previous dilution of MIC *C. parapsilosis*; (d) MIC of *C. parapsilosisi*.

all *C. neoformans* isolates were susceptible to fluconazole, whereas three isolates of *C. gattii* were susceptible dose-dependent and the remaining 21 were classified as susceptible (Table 1). MICs comparison of both methodologies demonstrated 100% categorical agreement for *C. neoformans* and *C. gattii*. The MICs for *C. gattii* in CLSI method ranged from 0.12–32 µg/ml and those found with FCM ranged from 1–32 µg/ml, whereas MICs for *C. neoformans* in the CLSI methods ranged from 0.25–4.0 µg/ml and FCM ranged from 1–8 µg/ml (Fig. 3). In general, lower MICs were obtained with the CLSI method compared to FCM, especially for *C. neoformans*, despite eight isolates showing MICs <1 µg/ml using the CLSI method (minimum dilution used for FCM) (Table 1).

The Spearman Correlation coefficient was used to compare the MICs obtained by the two methodologies and revealed a significant positive correlation coefficient, Pv = 0.001 obtained at the 0.01 level.

Discussion

Flow cytometry has been employed over the past few years for *in vitro* antifungal susceptibility testing (AST) in order to shorten the incubation time for slow growing molds [28] and providing greater accuracy in less time as the method analyzes larger number of cells allowing the detection of cell damage [20–22,25].

The AST methods used for *Cryptococcus* were standadized with *Candida* species that grow faster than members of the former species [15]. Therefore, FCM is of interest as it may overcome such limitations [20] of conventional procedures for *C. neoformans* and *C. gattii*. The current high price of the FCM equipment can be a limiting factor in applying the methodology in clinical laboratories. However, in the laboratories which routinely used FCM for diagnostic and other purposes, AST could be performed with a positive cost/benefit ratio, especially when compared to the commercial methods that can be fiscally prohibitive.

Table 1. Minimum inhibitory concentration (MIC) of fluconazole obtained by Clinical and Laboratory Standards Institute (CLSI) and flow cytometry and categorical correlation of *Cryptococcus neoformans* and *Cryptococcus gattii*.

Isolates	CLSI‡ MIC2§ (μg/ml)	FCM† MIC2 (μg/ml)	CLSI and FCM Correlation categorical**
C. neoformans			
LMM*1426	1.00	8.00	S
LMM1494	<1	2.00	S
LMM1433	4.00	4.00	S
LMM1434	1.00	<1.00	S
LMM1443	4.00	4.00	S
LMM1446	1.00	4.00	S
LMM1445	<1	1.00	S
LMM1450	<1	2.00	S
LMM1452	4.00	4.00	S
LMM1455	1.00	4.00	S
LMM1457	1.00	2.00	S
LMM1468	1.00	1.00	S
LMM1473	1.00	2.00	S
LMM1475	1.00	2.00	S
LMM1496	2.00	2.00	S
LMM1498	2.00	4.00	S
C. gattii			
LMM201	<1	1.00	S
LMM202	2.00	1.00	S
LMM244	<1	1.00	S
LMM253	<1	1.00	S
LMM272	1.00	1.00	S
LMM326	2.00	1.00	S
LMM330	<1	<1.00	S
LMM347	1.00	1.00	S
LMM362	2.00	1.00	S
LMM378	<1	<1.00	S
LMM384	1.00	1.00	S
LMM1430	4.00	1.00	S
LMM1436	2.00	2.00	S
LMM1422	2.00	4.00	S
LMM1425	32.00	32.00	SDD
LMM1429	32.00	32.00	SDD
LMM1431	2.00	2.00	S
LMM1432	2.00	8.00	S
LMM1435	32.00	32.00	SDD
LMM1437	4.00	4.00	S
LMM1438	4.00	8.00	S
LMM1441	1.00	8.00	S
LMM1463	1.00	8.00	S
LMM1493	1.00	8.00	S

S, susceptible; SDD, susceptible dose-dependent; *Laboratory Medical Mycology; ‡broth microdilution guidelines; §~50% optical density value of the growth control; †FCM, flow cytometry. **The Categorical classification for *Candida* sp. was used to interpret *Cryptococcus* spp. MICs obtained by CLSI and FCM as the breakpoints for *Cryptococcus* species are not established.

Different fluorochromes have been used in antifungal susceptibility tests with FCM, especially for *Candida* species. For the analysis of *Cryptococcus* spp., the fluorocromes propidium iodide (PI) and FUN-1 were used by Ramani et al. [29], Chaturvedi et al. [30] and Joung et al. [19]. So far, variable results have been obtained with the few strains of *C. neoformans* that have been analyzed with FCM using PI and FUN-1 [22,31,32]. Green et al. [31] obtained comparable results between microdilution method and FCM using PI, but the authors tested only one strain of *C. neoformans* among several isolates of *Candida* spp. and *Saccharomyces cerevisiae*. Pina-Vaz et al. [22] also compared both methodologies, analysing three *Cryptococcus* and 63 *Candida* isolates, and obtained comparable results with FCM using FUN-1 after 1 h of incubation in tests involving itraconazole, voriconazole and caspofungin. Ramani and Chaturvedi [32] tested the *in vtro* AST of 16 strains of *C. neoformans* to amphotericin B and FCZ with FCM using PI and obtained results comparable to those found with the CLSI procedures after 4 and 6 h of incubation for amphotericin B and FCZ, respectively. However, FCM required the use of one more step, i.e., the addition of sodium deoxycholate at the end of incubation to improve the permeability for PI. According to Rudensky et al. [25], sodium deoxycholate results in gelling of the yeast suspension during cytometer reading, so they proposed the use of acridine orange (AO) as the viability indicator of *Candida* spp.

Most antifungal susceptibilities studies with FCM are based on the mean channel fluorescence of the dead cells, as used by Ramani et al., with 50% increasing mean channel fluorescence for *Candida albicans* using PI [29]. However, another study demonstrated that a 30% increase in mean channel fluorescence was best correlated to the MICs of *Candida* spp. to FCZ [33].

In this study the MICs found with FCM were obtained by ~50% of AO negative cells count, comparing with the AO negative of the growth control for each isolate. Thus, MICs results were based on living cells, as used in the CLSI method. We obtained comparable results to those obtained with the CLSI method in our pilot study of *C. neoformans* and *C. gattii* susceptibility against FCZ with 9-h incubation. A considerable number of living cells was lost in the growth control (drug-free) during 9-h incubation at 200 rpm. Approximately 1700 positive AO cells were detected compared to the control prepared 30 min prior to acquisitions by FCM. Thus, the fast death of cryptococcal cells observed in 9 h was one of the reasons to choose counting AO negative cells to determine the MICs by FCM. Moreover, the use of AO eliminated the need for the additional sodium deoxycholate [25].

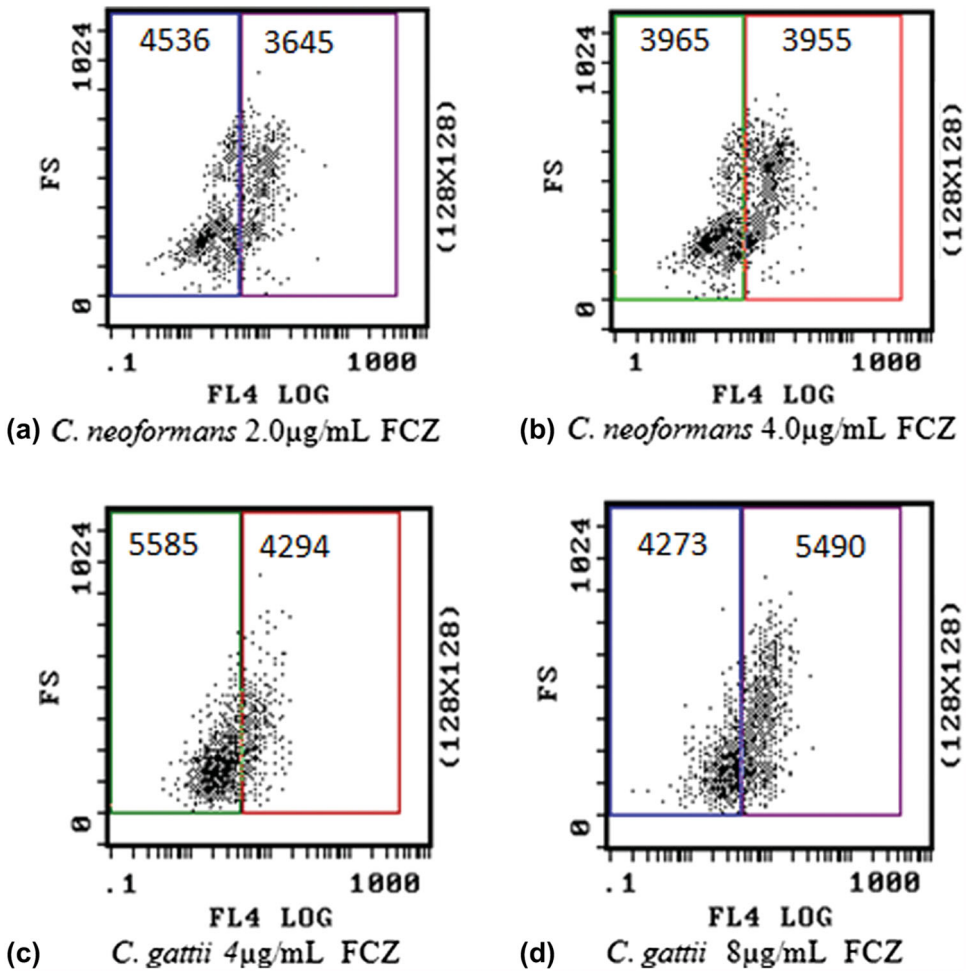

Figure 3. Dot plot of the corresponding MIC and the previous dilution for representative strains of *Cryptococcus neoformans* (LMM1433) and *C. gattii* (LMM1493): (a) previous dilution of MIC *C. neoformans*; (b) MIC of *C. neoformans*; (c) previous dilution of MIC *C. gattii*; (d) MIC of *C. gattii*.

Usually the FCM method to evaluate the *in vitro* fluconazole susceptibility using FUN-1 and PI provided rapid and reproducible results in 6–8 h for *Candida* species [32,33]. In contrast, our study determined 9 h was shortest incubation time for *C. neoformans* and *C. gattiii*, but 12, 19 and 24 h presented a variation of one dilution.

The MICs ranges obtained by CLSI and FCM methods were similar, although 0.12 µg/ml or 1 µg/ml is the lowest dilution in CLSI and FCM, respectively. This difference may have influenced the statistical analysis since eight strains had MIC <1 µg/ml with the CLSI method, but a significant positive correlation coefficient was still obtained. The range concentration of 1–64 µg/ml for FCM was used because in the categorical classification MICs $\leq$ 8 µg/ml indicate susceptible (S), 16–32 µg/ml susceptible dose-dependent (S-DD) and $\geq$64 resistant (R) strains [15]. Thus, when considered categorical classification, 100% agreement between both methodologies was observed for *C. neoformans* and *C. gattii* strains.

CLSI inoculum is small and variable (500–2,500 CFU/ml), while FCM method has the advantage of allowing the analysis of a large and constant number of cells, such as 10,000 cells (in this study), or 30,000 by Pina-Vaz et al. [22], yielding a more representative result. Thus, we consider inoculum size to be a critical factor for achieving reproducible results with isolates containing heterogeneous sub-populations of cells with different fluconazole susceptibilities, such as heteroresistance of *C. neoformans* described by Yamazumi et al. [34].

In conclusion, we present a rapid flow cytometry assay using acridine orange for *C. neoformans* and *C. gattii* susceptibility testing. Flow cytometry proved to be reproducible, with the additional advantage of analyzing a large and a constant number of cells, allowing comparative studies carried out on *Cryptococcus* isolates in less time (9 hours) than the standard CLSI M27-A3 method (72 h). The FCM method can expedite and improve the results of *in vitro* susceptibility tests of *C. neoformans* and *C. gattii*.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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