Comparison of Two Probes for Testing Susceptibilities of Pathogenic Yeasts to Voriconazole, Itraconazole, and Caspofungin by Flow Cytometry

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A cytometric approach to determine the susceptibilities of *Candida* spp. and *Cryptococcus neoformans* to voriconazole, itraconazole, and caspofungin is described. A total of 63 clinical isolates with different susceptibility patterns were exposed for 1, 2, 4, and 6 h to serial concentrations of each antifungal agent, followed by staining with two fluorescent probes: propidium iodide (PI) and FUN-1. FUN-1 was able to identify the susceptibility patterns of the assayed strains to the three agents after 1 h. PI penetrated a maximum of 50% of the cells treated with PI, at the highest concentration of caspofungin, 16 µg/ml, after 6 h of incubation (this percentage varied with the strain and was drug concentration and time of incubation dependent) and did not stain cells treated with high concentrations of either azole after 6 h. The use of FUN-1 appears to be an excellent fast and reliable alternative to the classical dilution method for determining the susceptibility of *Candida* spp. and *C. neoformans* to these three antifungal agents.

Prior to the introduction of the azoles, few therapeutic choices were available for fungemia, which limited the role of antifungal susceptibility testing (AFST). AFST could be useful in the selection of empirical treatment and for testing isolates from blood, from deep-seated infections, or from recurrent mucosal infections (8). The role of AFST is also important in light of the increase in the number of severe non-*albicans* *Candida* infections and the introduction of new broad-spectrum azoles and echinocandins. The use of standardized antifungal susceptibility methods has improved interlaboratory reproducibility. However, the ability of antifungal susceptibility testing to predict clinical outcome is still being elucidated for the new agent caspofungin; tentative breakpoints are still under discussion for voriconazole by the Clinical Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards). The CLSI microdilution method is labor and time-consuming and difficult to use in small clinical laboratories. Some alternatives have emerged, including colorimetric or fluorescent methods, which could improve definition of susceptibility patterns and or automation. Commercial antifungal methods also have been developed (6, 11, 13) that are easy to perform but also require a minimum of 24 to 48 h.

Flow cytometry has been described as an excellent tool for studying the susceptibility of different microorganisms, including fungi (9, 16, 20, 23). This methodology allows the timely determination of susceptibility patterns and can provide additional information regarding drug action and resistance mechanisms (17–19). An optimized cytometric assay to study the susceptibility of *Candida* spp. with incubation for 2 h with

amphotericin B and 4 h with fluconazole has been described recently and shows a good correlation with the CLSI method (2).

We evaluated here a cytometric protocol to determine the susceptibility of *Candida* spp. and *Cryptococcus neoformans* to the triazoles itraconazole and voriconazole and to the echinocandin caspofungin. The performance of the following two fluorescent markers was evaluated: propidium iodide (PI), a marker of cell death (it only penetrates cells with severe membrane lesions), and FUN-1, a probe that is converted by metabolically active fungi from a diffuse cytosolic pool (green-yellow fluorescence) to red cytoplasmic intravacuolar structures (10). Metabolically disturbed cells show an increase in the intensity of green-yellow fluorescence.

MATERIALS AND METHODS

Isolates. Sixty-three clinical strains of *Candida* spp. (26 *Candida albicans*, 4 *C. glabrata*, 5 *C. guilliermondii*, 7 *C. parapsilosis*, 16 *C. krusei*, and 5 *C. tropicalis*) and 3 *Cryptococcus neoformans* strains were isolated from blood, urine, lower respiratory tract, and spinal fluid specimens. *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258 were also studied. All clinical isolates were identified by using Vitek system (bioMérieux, Vercieux, France). Until testing, the yeasts were kept at –70°C in brain heart infusion broth (Difco Laboratories, Detroit, MI) with 5% glycerol. Each strain was subcultured twice on Sabouraud agar (Difco) prior to testing to confirm its purity and viability.

Determination of MICs. MICs of voriconazole (Pfizer, Groton, CT) and itraconazole (Jansen, Beerse, Belgium) were determined according to the NCCLS M27-A2 microdilution method guidelines (12); for caspofungin (Merck, Rahway, NJ), the same conditions described for azoles were used, but the MIC was defined as the lowest concentration in which a score of 0 (optically clear) was observed.

Flow cytometry analysis. (i) **Optimization of the cytometric protocol.** Yeast cells were cultured with shaking (200 rpm) at 35°C in Sabouraud broth until late log phase (as determined by a growth curve constructed from absorbance readings at an optical density of 600 nm). The cells were centrifuged, suspended in phosphate-buffered saline (Sigma) supplemented with 2% glucose (pH = 7.0), and counted in a Newbauer chamber. Suspensions containing 10⁶ cells/ml were incubated with shaking at 35°C with each antifungal agent for 1, 2, 4, and 6 h; the

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FIG. 1. Three-dimensional plots illustrating yeast cells of a typical susceptible strain to caspofungin stained with PI. (A) Ethanol-treated cells, all of the population stained with PI. (B) Viable cells, PI negative. (C) Cells treated with 1 μg of caspofungin/ml (four times the MIC) during 1 h (most of the population did not stain with PI). (D) Cells treated with 1 μg of caspofungin/ml for 6 h (26.6% of the cells were PI positive).

itraconazole and voriconazole concentrations were 0.125, 0.5, 1, 2, 4, and 8 $\mu\text{g}/\text{ml}$, and the caspofungin concentrations were 0.25, 1, 2, 4, 8, and 16 $\mu\text{g}/\text{ml}$.

(ii) **PI staining.** After the antifungal treatment, yeast cells (10^6) were washed to avoid quenching of fluorescence and stained with 1 μg of PI/ml (Sigma, St. Louis, MO) in the dark at room temperature for 30 min in HEPES solution (pH 7.2) supplemented with 2% glucose (GH solution) (16). Suspensions of untreated (drug-free) and killed cells with 70% ethanol were stained using the same conditions used with PI and used as controls.

(iii) **FUN-1 staining.** Antifungal treated and control cells (10^6) were washed and stained with 0.5 μM FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinoliniumiodide; Molecular Probes, Europe BV, Leiden, The Netherlands] in HEPES solution (pH 7.2) supplemented with 2% glucose (GH solution) in the dark at room temperature for 30 min (16). Suspensions of untreated (drug free) and cells treated with 1 mM sodium azide (Sigma) for 1 h were stained under the same conditions with FUN-1 and used as controls.

(iv) **Cytometry analysis.** After staining, ca. 30,000 cells were analyzed (Beckman Coulter XL-MCL flow cytometer with an argon 15-mV laser) at least twice for each cytometric reading. The cell scattergram, the autofluorescence, and the intensity of fluorescence at FL2 (yellow-green fluorescence, 575 nm) and at FL3 (red fluorescence, 620 nm) were recorded by using a logarithmic scale. For PI, the results were expressed as the percentage of positive cells showing high fluorescence at FL3; for FUN-1, the results were expressed as a staining index (SI), defined as the ratio between the mean fluorescence of treated cell suspensions and the corresponding value for the nontreated cells at FL2. The susceptibility phenotype was defined according to the lowest concentration of the antifungal agent that resulted in an SI above that of the control, that is an SI of >1 , after drug treatment (20). As described for itraconazole (12), MICs of ≤ 0.125 $\mu\text{g}/\text{ml}$ were considered susceptible, MICs between 0.25 and 0.5 $\mu\text{g}/\text{ml}$ were considered susceptible-dose dependent, and MICs of ≥ 1 $\mu\text{g}/\text{ml}$ were considered resistant. For voriconazole, MICs of ≤ 1 $\mu\text{g}/\text{ml}$ were considered suscep-

tible, MICs of 2 $\mu\text{g}/\text{ml}$ were considered susceptible-dose dependent, and MICs of ≥ 4 $\mu\text{g}/\text{ml}$ were considered resistant (newly established tentative breakpoints). Although, susceptibility breakpoints have not yet been established for caspofungin, strains that are inhibited by ≤ 1 $\mu\text{g}/\text{ml}$ could be considered susceptible (14). Whenever the SI was >1 after incubation with concentrations ≥ 1 $\mu\text{g}/\text{ml}$ for itraconazole, >1 $\mu\text{g}/\text{ml}$ for caspofungin, and ≥ 4 $\mu\text{g}/\text{ml}$ for voriconazole, the strains were considered resistant.

Viability assessment. The number of viable cells, both for treated and untreated suspensions, was determined by enumeration of CFU/ml on Sabouraud agar plates after plating 100 μl of serial dilutions and incubation at 35°C for 48 h.

Agreement between both methods. The categorical agreement between CLSI standards and determination by flow cytometry, after staining with FUN-1, was determined by comparing results for each isolate evaluated.

RESULTS

Untreated cells stained with PI showed a very low intensity of fluorescence and, in contrast, ethanol-treated cells showed a high red fluorescence. The two histograms are so dissimilar that the results were expressed as positive (dead cells, Fig. 1A) or negative (viable cells, Fig. 1B) for PI. *Candida* cells treated with both triazoles did not stain with PI, a finding which agrees with cell viability results; viability was not affected in these experimental conditions, even with the highest triazole concentrations after 6 h of incubation. Treatment with four times MIC of caspofungin during 1 h did not result in PI staining of yeast cells. Conversely, a growing percentage of cells become PI positive as the drug concentration and time of incubation

FIG. 2. (A and B) Percentage of PI-positive (PI+) cells (A) and nonviable cells (B) after exposure of *C. albicans*, an example of a susceptible strain, to different concentrations of caspofungin for 6 h.

increased (Fig. 2A). A maximum of 20% PI-positive cells were observed after 6 h (Fig. 1C and D), varying between strains (10 to 20%) and, when incubated with the highest concentration (16 µg/ml), this percentage could be a maximum of 50% after 6 h, varying between susceptible strains (30 to 50%). These effects were not observed on resistant strains to the drug. Nevertheless, after plating, the number of nonviable cells surpassed the number of PI stained cells on susceptible strains (Fig. 2B). After 6 h with the highest concentration of caspofungin used (16 µg/ml) and despite the fact that more than 90% of the cells were nonviable, approximately 50% of the cells were stained by PI (Fig. 2). This effect was also variable with the strains.

Yeast cells exposed to 1 mM sodium azide (used as a control of metabolic impairment) and stained with FUN-1 showed an increase in fluorescence intensity compared to nontreated cells corresponding to an SI of >1 (Fig. 3A). The effect was similar for susceptible strains after incubation with low drug concentrations (0.125 for itraconazole; 1 µg/ml for the other two), whereas this effect only occurred among resistant strains after incubation with the highest drug concentrations (Fig. 3). A dose-dependent and time-dependent increase in SI (SI > 1)

was seen with the three tested antifungals. These differences were evident after 1 h of incubation for the three antifungal agents, with an excellent correlation with NCCLS microdilution MIC results. The susceptibility phenotypes of the *Candida* and *Cryptococcus* species evaluated to itraconazole, voriconazole, and caspofungin by NCCLS and flow cytometry methods after FUN-1 staining are shown in Table 1.

DISCUSSION

Yeast represents at present the fourth leading organism causing septicemia in the United States, Europe, and Australia (7, 15, 22), in particular in intensive care units (4). The availability of rapid and reliable tools to determine the susceptibility of yeasts, namely, *Candida* and *Cryptococcus* spp., to antifungal agents is mandatory. In the last few years, the susceptibility to antifungal agents has been evaluated by several flow cytometry methods (16, 17, 18, 19, 20, 21, 23); these methods can save time and provide additional information regarding mechanisms of action and resistance of the drugs tested. We have already developed cytometric protocols to study amphotericin B, flucytosine, and fluconazole (16, 19), but

TABLE 1. Agreement between itraconazole, voriconazole, and caspofungin phenotypes of *Candida* spp. and *C. neoformans* as determined by NCCLS M27-A2 and flow cytometry (FC: FUN-1 staining) methods

Strain	Flow cytometry phenotype (no. of isolates)																Total isolates
	Itraconazole ^a						Voriconazole ^b						Caspofungin ^c				
	NCCLS phenotype			FC phenotype			NCCLS phenotype			FC phenotype			NCCLS phenotype		FC phenotype		
	S	S-DD	R	S	S-DD	R	S	S-DD	R	S	S-DD	R	S	R	S	R	
<i>C. albicans</i>	2	1	23	2	1	23	6	0	20	6	0	20	24	2	24	2	26
<i>C. glabrata</i>	0	3	1	0	3	1	3	0	1	3	0	1	3	1	3	1	4
<i>C. guilliermondii</i>	1	4	0	1	4	0	5	0	0	5	0	0	0	5	0	5	5
<i>C. parapsilosis</i>	5	1	1	5	0	2	7	0	0	7	0	0	1	6	1	6	7
<i>C. krusei</i>	1	14	1	1	14	1	15	1	0	15	1	0	1	15	1	15	16
<i>C. tropicalis</i>	1	0	4	1	0	4	1	1	3	1	1	3	4	1	4	1	5
<i>C. neoformans</i>	2	1	0	2	1	0	3	0	0	3	0	0	0	3	0	3	3
Total	12	24	30	12	23	31	40	2	24	40	2	24	33	33	33	33	66

^a The flow cytometry phenotype for itraconazole was defined according to the SI, calculated as the ratio between the intensity of the fluorescence of treated and of nontreated cells. If SI > 1 after incubation with 0.125 µg of itraconazole/ml, the strain was considered susceptible (S); if SI > 1 only after incubation with 0.25 to 0.5 µg of itraconazole/ml, the strain was considered susceptible-dose dependent (S-DD); and if SI > 1 only after incubation with ≥ 1 µg of itraconazole/ml, the strain was considered resistant (R).

^b The flow cytometry phenotype for voriconazole was defined according the SI, calculated as the ratio between the intensity of the fluorescence of treated and of nontreated cells. If SI > 1 after incubation with voriconazole at ≤ 1 µg/ml, the strain was considered susceptible (S); if SI > 1 only after incubation with 2 µg of voriconazole/ml, the strain was considered susceptible-dose dependent (S-DD); if SI > 1 only after incubation with voriconazole at ≥ 4 µg/ml, the strain was considered resistant (R).

^c The flow cytometry phenotype of caspofungin was defined according the SI, calculated as the ratio between the intensity of fluorescence of treated and of nontreated cells. If SI > 1 after incubation with caspofungin at ≤ 1 µg/ml, the strain was considered susceptible (S); if SI > 1 only after incubation with caspofungin at > 1 µg/ml, the strain was considered resistant (R).

each antifungal class may require a different protocol that takes into consideration its molecular characteristics and mechanisms of action. Cytometric methodology has also been evaluated for testing itraconazole with acridine orange, ethidium bromide, and fluorescein diacetate, but these methods require 8 to 24 h to yield results (9). A flow cytometry assay has recently been described to study *Aspergillus fumigatus* versus voriconazole using PI as the marker of fungicidal activity (21). However, although voriconazole may be fungicidal for *Aspergillus* spp., it is only fungistatic against yeasts. A single study has used cytometric methodology with caspofungin, using confocal microscopy after staining *Candida* cells with FUN-1, to study its effect on planktonic *Candida* cells versus cells on biofilms (1).

Due to the lack of fast and reliable cytometric assays, we optimized our cytometric methodology to study the susceptibility of pathogenic yeasts to these three antifungal agents. Two fluorescent probes, previously optimized by us (16), PI and FUN-1, were compared.

In the present study, the performance of the PI probe was limited for evaluating the two triazoles in vitro activities, similar to our results for fluconazole and flucytosine (16). This effect was expected since they are only fungistatic drugs. Raman et al. were able to stain with PI yeast cells treated with fluconazole, but only after using a detergent of the membrane, sodium deoxycholate, that facilitates the diffusion of PI into the cells (20). Although it was possible to appreciate the fungicidal effect of caspofungin against some *Candida* spp., the percentage was variable and low with the PI probe, mainly after a short incubation time. Caspofungin has in vitro fungicidal activity against certain strains of *Candida* spp. (5). Since PI did not penetrate the yeast cells quickly, we could conclude that the fungicidal effect of caspofungin was not initiated by

producing primary cytoplasmic membrane lesions but perhaps by inhibiting the synthesis of fungal cell wall (3). In contrast, when permeability occurs after a short incubation time (15 min), the primary effect could be due to membrane lesions (17, 18). However, it has been demonstrated that although cells treated with amphotericin B were dead, there was no diffusion of PI into these dead cells (16).

After uptake and accumulation in the cell cytoplasm, FUN-1 is converted only by metabolically active yeasts. Sodium azide (1 mM) inhibits the respiratory metabolism, rendering cells unable to process the probe (10). The intensity of fluorescence at FL2 of azide-treated cells was significantly higher than that of nontreated cells, corresponding to an SI of >1. Accordingly, an SI of >1 after antifungal treatment indicated a serious impairment of yeast metabolism, having the advantage of being an earlier marker of cell damage, before cell death occurs by a fungicidal agent. For susceptible strains, the fluorescence intensity of cells treated with low drug concentrations increased compared to control cells (SI > 1). This effect only occurred in the resistant strains when challenged with the highest drug concentrations (4 to 16 µg/ml); the effect was similar to that observed previously with fluconazole (14) and flucytosine (19, 14). We were able to identify itraconazole categorical endpoints after 1 h of incubation with this agent; the overall time in other studies for this incubation period is 4 to 6 h for yeasts (20, 2) and 2 to 3 h for *A. fumigatus* (21).

Using the two fluorescent probes, we were then able to achieve a better understanding of what is happening to the yeast cells after incubation with serial drug concentrations and have obtained a kinetic perspective of the activity of caspofungin against yeasts. In conclusion, flow cytometry analysis with FUN-1 proved to be an excellent tool and superior to the PI probe for studying the susceptibility of *Candida* spp. and *C.*

neoformans to these three drugs. Once again, the cytometric approach to susceptibility testing has proven to have potential as an important tool in research and clinical laboratories to provide in vitro results for clinical isolates in a timely manner. The reproducibility and clinical utility of this methodology, especially for caspofungin, is yet to be determined.

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