ORIGINAL ARTICLE

Cytometric approach for a rapid evaluation of susceptibility of *Candida* strains to antifungals

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Objective   To achieve a fast and reliable determination of the susceptibility of *Candida* strains to amphotericin B (Am B), fluconazole (Flu) and 5-fluorocytosine (5-FC), using cytometric methods as an alternative to the classical dilution method.

Methods   Twenty-three clinical isolates of *Candida* with different susceptibility patterns were treated for 1 h with two concentrations each of Am B (2 and 8 mg/L), Flu (8 and 64 mg/L) and 5-FC (4 and 32 mg/L), followed by staining with three different fluorochromes, under conditions previously defined through an optimisation study. These were 1 mg/L propidium iodide (PI)/10^6 cells for 30 min at 30 °C (a marker that only penetrates cells with severe lesions of the membrane); 0.5 μM FUN-1/10^6 cells for 30 min at 30 °C (a fluorescent probe which after entering the yeast cell is converted, by metabolically active yeasts, from a diffuse cytosolic pool with a yellow-green fluorescence into red cylindrical intravacuolar structures) and 0.25 μM of JC-1/10^6 cells for 15 min at 37 °C (a monomer that changes reversibly from green to red the J-aggregates, with the increased membrane potential). About 50 000 yeast cells were analysed by flow cytometry (FCM), at FL3 (red, 620 nm) for PI and FL2 (yellow–green, 575 nm) for FUN-1 and the ratio of FL3 to FL1 was determined (red, 620 nm/green, 525 nm) for JC-1; 200 cells of each suspension were also analysed by epifluorescence microscopy (EPM). Viability studies were performed in parallel to count the number of colony forming units.

Results   Susceptible (S) strains exposed to Am B and stained with JC-1 showed a dose–dependent decrease in the mitochondrial potential, i.e. a decreased ratio between red/green fluorescence by FCM and a decrease in J-aggregates by EPM. Neither FUN-1 nor PI was useful in the study of Am B activity. Susceptibility to Flu and 5-FC could be detected with FUN-1 staining: metabolic changes were detected by an increase in yellow–green intensity of fluorescence by FCM or a decrease of cylindrical intravacuolar structure formation by EPM, although no decrease in total viability was registered. Staining with JC-1 could predict resistance to both drugs, but did not allow distinction between sensitive dose-dependent strains (S-DD) or intermediate (I) resistance to Flu or 5-FC, respectively, from S strains. PI did not stain *Candida* cells treated with Flu or 5-FC under our experimental conditions.

Conclusion   Susceptibility patterns of *Candida* strains to Am B can be determined by using JC-1, and to Flu and 5-FC by using FUN-1. PI was not a useful probe with which to study the effect of such antifungals under the conditions described here.

Keywords   *Candida* spp., antifungal susceptibility testing, cytometry, FUN-1, propidium iodide, JC-1

Accepted 12 June 2001

Clin Microbiol Infect 2001; 7: 609–618

INTRODUCTION

The increasing incidence of opportunistic severe fungal infections has greatly enhanced the interest in novel methods for in

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vitro antifungal susceptibility testing. The standardised methodology recommended by the National Committee for Clinical Laboratory Standards (NCCLS) M27-A [1], represents a significant step in the development of a reproducible reference testing method. Nevertheless this macrodilution technique is cumbersome and labor intensive. Additionally, its correlation with the clinical outcome is still under evaluation [2]. During the last two decades, flow cytometry (FCM) has proved to be a powerful research tool, e.g. to study the effect of various compounds on microbes [3–6]. Fluorescent dyes have been used to study drug–cell interactions [7–9]. Measurement of alterations in fungal cell viability can be performed with great

precision using various fluorescent probes which have specific cellular affinities [10]. Using different probes it is possible to appreciate pre-lethal changes, either from a metabolic or a morphological point of view, which may predict the susceptibility pattern to antifungal drugs.

The choice of an ideal probe, and of the optimal experimental conditions for its use, is mainly conditioned by the biochemical properties of the antifungal drugs.

In this study three different probes were used. Propidium iodide (PI) is a probe often used to stain non-viable cells. Dead or dying cells with injured membranes can incorporate PI [11] which stains the nucleic acids [12]. The second probe, FUN-1 [2-choro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1 phenylquinolinium iodide], was recently developed by Molecular Probes (Leiden, the Netherlands) as an indicator of yeast cell viability. Viable cells loaded with FUN-1 process and concentrate it within their vacuoles, thereby generating red cylindrical intravacuolar structures (CIVS) [13]; in non-viable cells, FUN-1 stays in the cytosol showing a diffuse yellow–green fluorescence and no CIVS. JC-1 (5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide), which is a fluorescent probe able to enter selectively the mitochondria in a monomeric form, emits a green fluorescence [14,15]. The colour changes from green to red, forming the J-aggregates, as the mitochondrial membrane becomes more polarised [16].

Our objective was to define the ideal fluorescent probes and the conditions for their use in susceptibility testing of *Candida* spp. to the three most important antifungal agents, using cytometric methods.

MATERIALS AND METHODS

Yeast strains

The *Candida* strains used are listed in Table 1. All the yeasts were clinical isolates, identified to species level using API 32C (BioMérieux, Vercieux, France). The strains were kept frozen in Brain–Heart Broth (Difco Laboratories, Detroit, MI, USA) with 5% glycerol at $-70\,^{\circ}\text{C}$ until testing. For each experiment, the yeasts were subcultured twice on Sabouraud agar (Difco) to assess the purity of the culture and its viability.

To render the cells non-viable, treatment with 70% ethanol for 10 min was used.

Antifungal drugs

Amphotericin B (Am B) (Sigma, St Louis, MO, USA), fluconazole (Flu) (Pfizer, Groton, CT, USA) and 5-fluorocytosine

Table 1 *Candida* strains studied and MIC values (μg/mL) to amphotericin B (AM-B), fluconazole (Flu) and 5-fluorocytosine (5-FC) determined by NCCLS protocol (M27-A)

Strains	Isolates from	MIC Am B[a] (M27-A)	MIC Flu[a] (M27-A)	Phenotype Flu	MIC 5-FC[a] (M27-A)	Phenotype[a] 5-FC
C. albicans SCO	vaginal	0.5	1	S	0.5	S
C. albicans HV18	bronchial wash	0.25	1	S	1	S
C. albicans HV22	bronchial wash	0.25	16	S-DD	4	S
C. albicans M44	vaginal	0.5	32	S-DD	16	I
C. glabrata HV94	stool	0.25	>64	R	16	I
C. glabrata HV91	bronchial wash	0.5	1	S	1	S
C. krusei H9	blood	0.25	64	R	>64	R
C. krusei HV10	blood	0.5	>64	R	16	I
C. tropicalis HV28	mouth	16	8	S	1	S
C. lusitanea H22	mouth	16	0.25	S	0.5	S
C. albicans H65	blood	0.5	>64	R	0.5	S
C. albicans H37	bronchial wash	0.5	>64	R	1	S
C. glabrata H36	blood	0.25	4	S	1	S
C. glabrata H30	blood	0.5	>64	R	>64	R
C. lusitanea H50	urine	0.5	1	S	1	S
C. krusei H11	blood	1	>64	R	>64	R
C. parapsilosis H5	urine	0.5	4	S	4	S
C. parapsilosis H6	blood	1	>64	R	>64	R
C. tropicalis H18	blood	0.5	4	S	1	S
C. tropicalis H25	urine	2	4	S	2	S
C. tropicalis 13803	ATCC	0.5	4	S	1	S
C. albicans 90028	ATCC	0.5	0.5	S	1	S
C. krusei 6258	ATCC	0.5	64	R	8	I

Classification of the strains according to the same protocol in susceptible (S), resistant (R), susceptible dose-dependent (S-DD) and intermediate (I).
[a]mg/L.

(5-FC) (Sigma) were prepared as recommended by the NCCLS protocol M27-A [1], and frozen until use.

Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the assayed antifungals for each isolate was determined according to the NCCLS protocol M27-A microdilution method [1], after 48 h of incubation.

Incubation with the antifungal drugs

Yeast cells were cultured at 35 °C in Sabouraud broth until late log phase (as determined by a growth curve constructed from absorbance readings at OD = 600 nm), with shaking (200 r.p.m.). The cells were centrifuged, suspended in phosphate-buffered saline (PBS) (Sigma) supplemented with 2% glucose, pH = 7.0, and counted in a Neubauer chamber. Suspensions containing 10^6 cells/mL were incubated with the antifungal drugs for 1, 2 and 4 h at 35 °C, with shaking (200 r.p.m.). Two concentrations of each drug (around the corresponding MIC breakpoints) were used: Amp B, 2 and 8 mg/L; Flu, 8 and 64 mg/L; 5-FCM, 4 and 32 mg/L.

Optimisation of staining methods for flow cytometry and epifluorescence microscopy assays

Cell counts

Suspensions with 10^3–10^7 cells/mL, counted in a Neubauer chamber, were tested. After this, a suspension of 10^6 cells/mL was always used for cytometric studies.

Propidium iodide

Suspensions of untreated cells were incubated for 30 min in the presence of 1.0, 2.5, 5.0 and 10.0 μg PI (Sigma)/10^6 cells, in HEPES solution, pH = 7.2, supplemented with 2% glucose (GH solution), in the dark, at room temperature. Yeast cells killed by 20 min in 70% ethanol were used as a control for the staining, after two previous washings with HEPES solution. Using 1 mg/L PI, we also studied suspensions of ethanol-exposed cells, stained for up to 180 min

FUN-1

Suspensions of 10^6 viable cells/mL in GH solution were incubated in the presence of 0.1–10.0 μM of FUN-1 [2-choro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1 phenylquinolinium iodide] (Molecular Probes, Leiden, the Netherlands). Using 0.5 μM FUN-1/10^6 cells/mL, we evaluated the variation of staining time up to 180 min. Viable *Candida* cells were also incubated for 1 h with 1 mM sodium azide (Sigma), an inhibitor of yeast metabolism, as a control for FUN-1 staining [13,17].

JC-1

Viable yeast cells were incubated with JC-1 (5,5′, 6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) (Molecular Probes), at concentrations ranging from 0.1 to 5.0 μM/10^6 cells/mL for 15 min, at 35 °C, in PBS supplemented with 2% glucose. A kinetic study of the staining was performed using 0.25 μM of JC-1/10^6 cells/mL, with exposure times up to 60 min Yeast cells were also incubated for 1 h with 100 μM of valinomycin (Sigma) (a K^+ ionophore known to reduce mitochondrial membrane potential) [15] and washed twice with PBS previous to staining with JC-1, as a control for the staining.

Viability assessment

The number of viable cells, both in treated and untreated suspensions, was determined by counting the number of colony forming units (CFU)/mL on Sabouraud agar plates, plated with 100 μL serial dilutions. Also, the assessment of the toxicity of the fluorochrome staining (1 mg/L of PI for 30 min, 0.5 and 1 μM of FUN-1 for 30 min and 0.25 μM of JC-1 for 15 min) was evaluated by CFU counts.

Staining protocol for yeast cells treated with antifungals

After treatment with the antifungals, as described above, yeast cell suspensions were washed to avoid quenching of fluorescence [18] and stained with 1 μg PI/10^6 cells for 30 min at room temperature, 0.5 or 1 μM of FUN-1/10^6 cells, or with 0.25 μM of JC-1/10^6 cells for 15 min at 37 °C. All the incubations were carried out in the dark.

A kinetic FCM study of PI and FUN-1 loading (up to 30 min) was performed with Am B-treated cells, i.e. a continuous measurement of the intensity of fluorescence with time.

Flow cytometry

The cell suspensions were analysed in a Beckman Coulter XL-MCL flow-cytometer with an argon 15 mV Laser, after an optimisation protocol. The cell scattergram [forward scatter (FS) and side scatter (SS)], the autofluorescence (without fluorocrome) and the intensity of fluorescence at FL1 (green fluorescence, 525 nm), at FL2 (yellow–green fluorescence, 575 nm) and at FL3 (red fluorescence, 620 nm) were recorded using a logarithmic scale. The results are expressed as follows: for PI, as a percentage of cells showing high fluorescence at FL3; for FUN-1, a staining index (SI), defined as the ratio between the mean fluorescence of treated cell suspensions and the corresponding value for the control (viable) cells at FL2; for JC-1, a ratio between the mean values of fluorescence at FL3 and FL1, calculated for each antifungal treated-suspension

and compared with the corresponding value for the untreated cells.

Each assay was run in duplicate.

Epifluorescence microscopy (EPM)

The yeast suspensions were observed under an epifluorescence microscope (Laborlux K, Leica, Germany) fitted with a mercury 50-W lamp, a BP 450–490 nm excitation filter and an LP 515-nm emission filter. Aliquots of each suspension were placed between slide and cover slip, after mixing with an anti-fading reagent (20 μL of the suspension + 20 μL of anti-fading reagent) (Vector Laboratories, Burlingane, CA, USA). For PI we looked for diffuse red staining of the cells; with FUN-1 the percentage of cells showing intravacuolar structures (CIVS) was determined; and with JC-1 we looked for the presence of J-aggregates.

Fluorimetry

GH solutions with FUN-1 (0.5 μM) or PI (1 μg/mL), either with or without Am B (8 mg/L) were analysed by fluorimetry (Spectrofluorometer FP-770 Jasco).

RESULTS

MIC corresponding to the different antifungals, for the strains tested strains are shown in Table 1. According to the interpretation guidelines for in vitro susceptibility testing of *Candida* isolates [1], the strains were classified as: susceptible (S) to Flu and 5-FC whenever MIC values were ≤8 mg/L and ≤4 mg/L, respectively; susceptible-dose dependent (S-DD) to Flu whenever MIC values were between 16 and 32 mg/L; intermediate to 5-FC, when MIC values were between 8 and 16 mg/L; and resistant (R) whenever MIC were ≥64 mg/L for Flu and ≥32 mg/L for 5-FC. No breakpoints are yet defined for Am B. Twenty-one strains showed a low MIC value and two strains showed a high MIC value (*Candida tropicalis* HV28 and *Candida lusitanea* H22) to Am B (see Table 1).

CFU counts showed that treatment with Am B for 1 h was highly fungicidal for the strains with low MIC, being considerably less active upon strains with higher MIC values. Conversely, Flu and 5-FC treatment showed a very minor effect upon cell viability for all the strains (Table 2 shows CFU counts for each susceptibility pattern to the different antifungals). Treatment with ethanol and valinomycin rendered most cells non-viable (Table 2). However, sodium azide, at 1 mM concentration, did not affect the viability of most of the cells (Table 2).

A concentration of 10^6 *Candida* cells/mL proved to be the most adequate for flow cytometry assays. The time required to obtain a representative scatter diagram was too long when using

Table 2 Values of colony forming units (CFU)/mL for three strains representative of each pattern of susceptibility to the different antifungals, after 1-hour treatment. H9 was susceptible to amphotericin B (AM B) and resistant to fluconazole (Flu) and 5-fluorocytosine (5-FC); HV28 was a strain with high MIC to Am B and susceptible to Flu and 5-FC; M44 was a strain susceptible dose-dependent to Flu and intermediate to 5-FC. The CFU after exposure to valinomycin (valino), ethanol 70% and sodium azide 1 mM are also represented

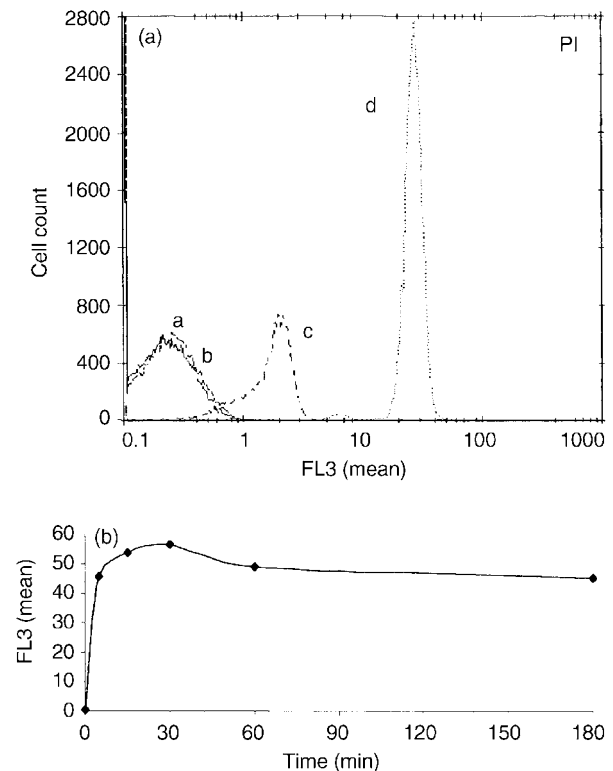|	H9	M44	HV28
Viable cells	100%	100%	100%
valino 100 μM	6.3%	5.4%	3.4%
ethanol 70%	0.0%	0.0%	0.0%
azide 1 mM	90.4%	91.4%	89.2%
Am B 2 mg/L	0.0%	–	84.9%
Am B 8 mg/L	0.0%	–	38.4%
Flu 8 mg/L	96.4%	93.6%	125.2%
Flu 64 mg/L	94.7%	82.3%	122.5%
5-FC 4 mg/L	98.9%	84.4%	120.3%
5-FC 32 mg/L	94.6%	112.2%	118.3%

Figure 1 (a) Flow cytometry of *Candida albicans* SCO cells stained with propidium iodide (PI) and analysed at FL3 (620 nm) showing: (trace a) autofluorescence of untreated (viable) cells; (trace b) autofluorescence of ethanol-treated (dead) cells; (trace c) fluorescence of untreated (viable) cells stained with 1 mg/L of PI for 30 min; and (trace d) fluorescence of ethanol-treated (dead) cells stained with 1 mg/L of PI for 30 min. (b) Kinetics of the staining of ethanol-treated (dead) cells of *Candida albicans* SCO with 1 mg/L of propidium iodide during 180 min.

lower cell concentrations, i. e. 10^3-10^5 cells/mL; thus 180 min were needed to analyse 8000 cells (data not shown). Conversely, when assaying 10^7 cells/mL, the yeasts showed a marked tendency to aggregate and interrupt the liquid flow through the detector unit.

The results with PI indicate that $1 \mu g/10^6$ cells is the optimal concentration for this fluorescent probe; stained viable cells show a fluorescence level similar to that of the autofluorescence and a clear distinction between viable and ethanol-killed cells is achieved (Figure 1a and Table 3). The intensity of PI staining (under the above mentioned conditions) of *Candida* cells killed by 10 min in 70% ethanol, increased during the initial 30 min, the intensity of the staining remaining constant afterwards (Figure 1b). Cells treated with ethanol showed a high permeability to the dye (i.e. >90% of cells stained) and were found to be dead by CFU counting.

As a concentration of $0.5 \mu M$ FUN-1/10^6 cells still provided good staining properties, it was chosen for further flow cytometry testing (Figure 2a,b). An increase in the intensity of fluorescence was evident when analysing cells exposed to sodium azide (Figure 2b). With the above indicated fluorochrome concentration, viable cells showed a quick increase in fluorescence during the first minutes, being maximum at

Table 3 Values of intensity of fluorescence at FL3 (620 nm) determined by flow cytometry with increased concentrations of propidium iodide (PI) for 30 min

PI (mg/L)	Viable[a] (FL3)	Dead[b] (FL3)
0	0.252	0.344
1.0	0.297	43.700
2.5	0.450	43.100
5.0	0.569	46.900
10.0	0.810	89.400

[a]Viable cells indicates non-treated cells; [b]dead cells indicates ethanol-treated cells.

30 min, which was immediately followed by a slight decrease (Figure 2c). After 30 min, FUN-1 staining stabilised, at least during the following 3 h (Figure 2c). However, for EPM, cells treated with FUN-1 at $1 \mu M/10^6$ cells/mL showed bigger cylindrical orange-red fluorescent CIVS, making the observation easier. CIVS were evident only in viable cells; conversely, an extremely bright diffuse yellow-green fluorescence was present in azide-exposed cells (not shown), under EPM.
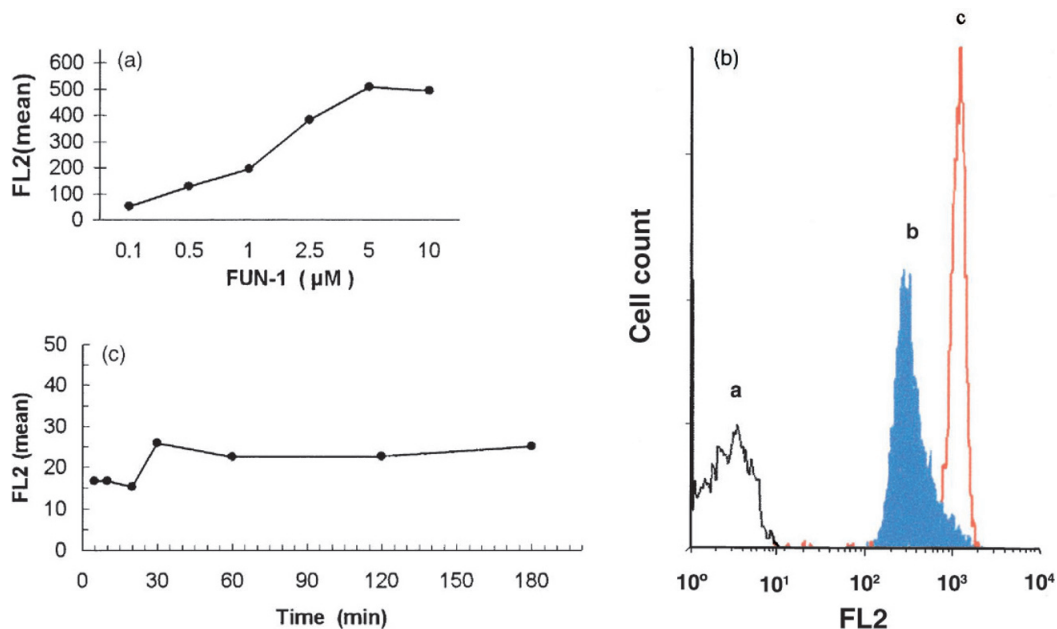

Figure 2 (a) Intensity of fluorescence of untreated (viable) cells of *Candida albicans* SCO after staining with different concentrations of FUN-1 for 30 min. **(b)** Flow cytometry of *Candida albicans* SCO cells stained with FUN-1 and analysed at FL2 (575 nm) showing: (trace a) autofluorescence of untreated (viable) cells; (trace b) fluorescence of untreated (viable) cells stained with 0.5 μM of FUN-1 for 30 min; and (trace c) fluorescence of yeast cells treated with 1mM of sodium azide and stained with 0.5 μM of FUN-1 for 30 min. **(c)** Kinetics of the staining of untreated (viable) cells of *Candida albicans* SCO with 0.5 μM of FUN-1 for 180 min.

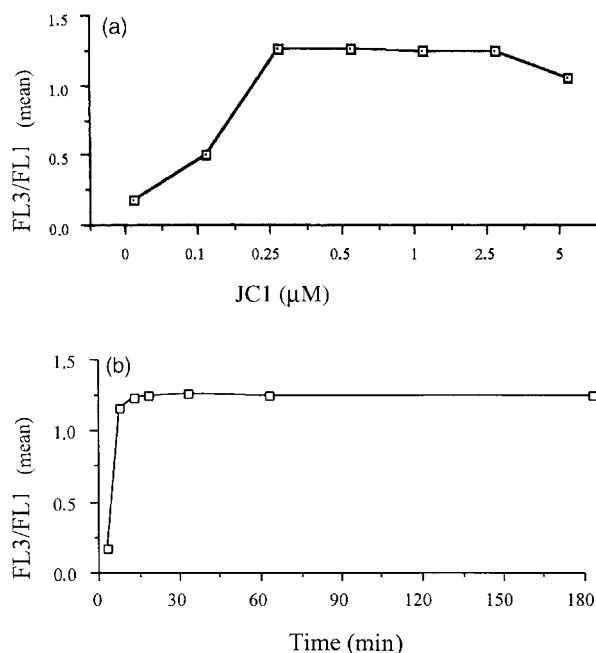Table 4 Values of the ratio between the intensity of fluorescence at FL3 (625 nm) and FL1 (525 nm) determined by flow cytometry of untreated cells (viable cells) and after 1 h treatment with valinomycin (valino), amphotericin B (AM B), fluconazole (Flu) and 5-fluorocytosine (5-FC) after staining with JC-1. The three strains represented a typical susceptibility pattern to each antifungal: H9 was susceptible to Am B and resistant to Flu and 5-FC; HV28 was a strain with a high MIC value to Am B and susceptible to Flu and 5-FC; M44 was a strain susceptible dose-dependent to Flu, and intermediate to 5-FC

	H9	M44	HV28
Viable cells	1.289	1.339	1.287
Valino 100 μM	0.507	0.623	0.611
Am B 2 mg/L	0.484	–	1.346
Am B 8 mg/L	0.700	–	1.353
Flu 8 mg/L	1.497	0.534	0.584
Flu 64 mg/L	1.607	0.447	0.559
5-FC 4 mg/L	2.019	0.544	0.469
5-FC 32 mg/L	1.788	0.445	0.401

Figure 3 (a) Intensity of fluorescence of FL3 (620 nm): FL1 (525 nm) of untreated (viable) cells of *Candida albicans* SCO after staining with different concentrations of JC-1 for 15 min. **(b)** Kinetics of the staining of untreated (viable) cells of *Candida albicans* SCO with 0.25 μM of JC-1 for 15 min.

With JC-1, 0.25 μM/10^6 cells/mL was the minimum concentration that could stain the yeast cells (Figure 3a), with 15 min being the optimal incubation time (Figure 3b). The exposure of yeast cells to valinomycin resulted in a decrease in the mean intensity of the ratio of FL3 to FL1, in comparison with untreated, viable cells (Table 4). EPM analysis showed two or three red aggregates per untreated yeast cell, the J-aggregates, which were not present after incubation with valinomycin.

After treatment with Am B the yeast cells of the different strains showed a slight decrease in the forward light scatter, indicating a slight decrease in size. Although more than 99% of cells of strains with a low MIC value to Am B were dead, neither PI nor FUN-1 was able to stain them. Conversely, such cells showed less intensity of staining by FCM than untreated yeasts (Figure 4). Under EPM, no cells were stained by PI nor did any show CIVS. Thus, a reduced loading of PI and FUN-1 was seen in both FCM and EPM in Am B-treated cells. The reduction in staining did not result from interference of the antifungal with the dyes, as deduced from the observation that the spectrophotometric spectra of both probes were not altered by the presence of Am B (data not shown).

With JC-1, a decrease in the mean value of FL3 : FL1 was seen only on strains with low MIC values (see strain H9 as a representative example on Table 4) and the J-aggregates were not present under EPM.

After Flu and 5-FC treatment PI did not enter the cells in any of the strains tested. However FUN-1 staining allowed a clear distinction between the S and the R strains to both drugs (Figures 5 and 6). S strains to Flu and 5-FC showed an increase in the mean intensity of fluorescence at FL2 (see strain HV28 as a representative strain on Figures 5 and 6), with a corresponding decrease in the number of cells with CIVS (not shown), despite the cells being able to grow (Table 2). R strains showed a staining index by FC similar to viable cells (SI > 1) (see strain H9 as a representative strain on Figures 5 and 6). Correspondingly, under EPM more than 95% of cells showed CIVS, which is in accordance with the viability testing (Table 2). S-DD strains to Flu and I strains to 5-FC presented a SI < 1 with the lower concentrations tested; however, a SI > 1 was seen with the highest drug concentration (Flu 64 mg/L or 5-FC 32 mg/L) (see strain M44 as a representative strain on Figures 5 and 6).

Strains classified as either S, S-DD, or I to Flu and 5-FC, respectively, and stained with JC-1 showed a decrease in the mean value of FL3 : FL1, compared to untreated cells (see a representative strain in Table 4). Thus no distinction between such susceptibility patterns was possible. With R strains no decrease in the ratio FL3 : FL1 was detected (see strain H9 as a representative on Table 4).

One hour was found to be an adequate incubation period, the results with longer incubation times (2 and 4 h) being similar (not shown).
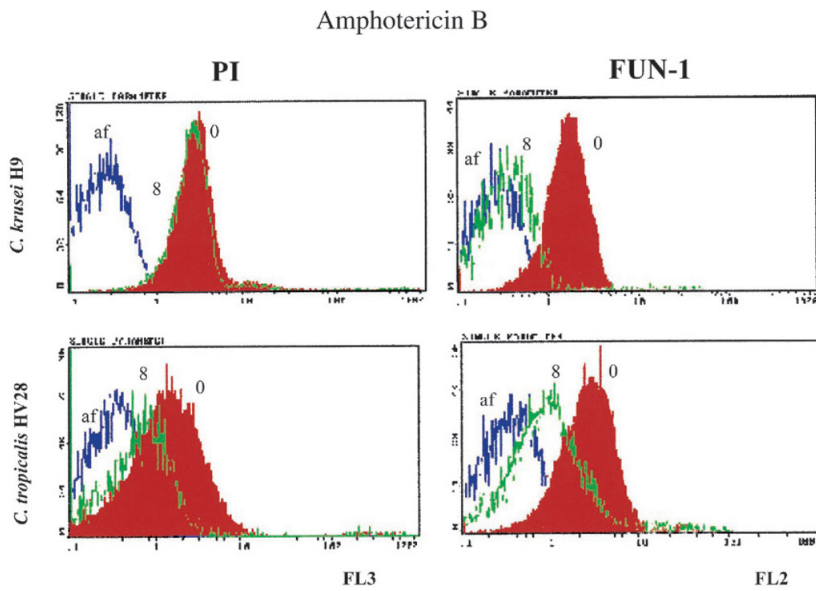
Amphotericin B

Figure 4 Flow cytometry of strain *Candida krusei* H9 (an example of a strain with low MIC value to Am B) and *Candida tropicalis* HV28 (an example of a strain with high MIC value to Am B) after treatment with 0 and 8 mg/L of Am B (0 and 8 on the figure) for 1 h and stained with propidium iodide (PI) or FUN-1. The autofluorescence (af) of the cells is also represented.
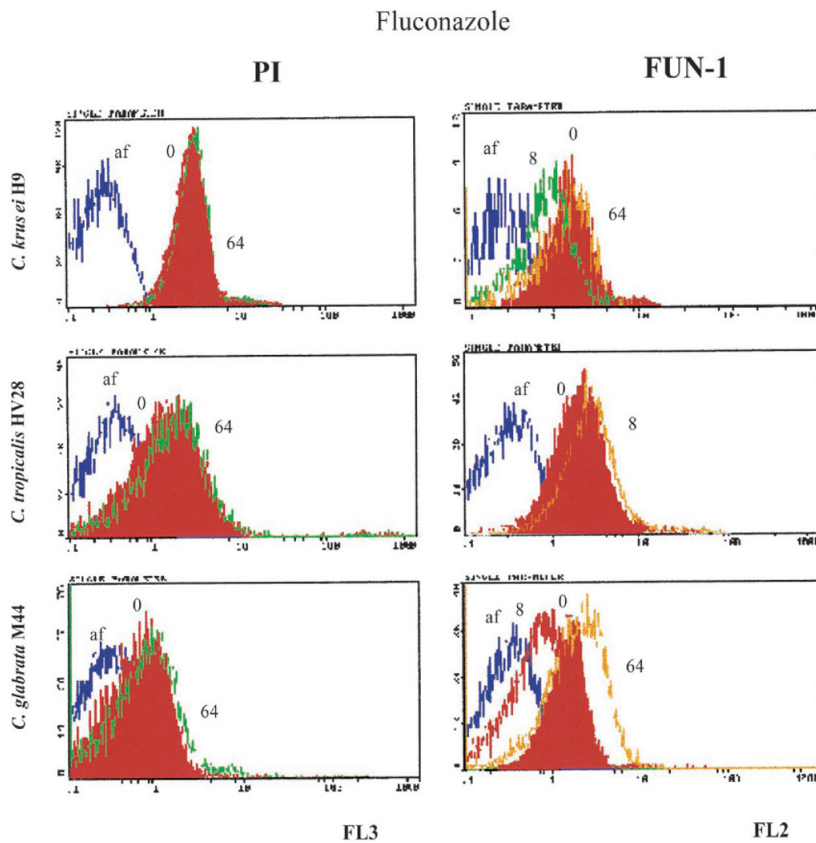
Fluconazole

Figure 5 Flow cytometry of the strain *Candida krusei* H9 (an example of a resistant strain to Flu and *Candida tropicalis* HV28 (an example of a susceptible strain to Flu) and *Candida glabrata* M44 (an example of a susceptible-dose dependent strain to Flu) after treatment with 0, 8 and 64 mg/L of Flu (0, 8 and 64 on the figure) for 1 h and stained with propidium iodide (PI) or FUN-1. The autofluorescence (af) of the cells is also represented.
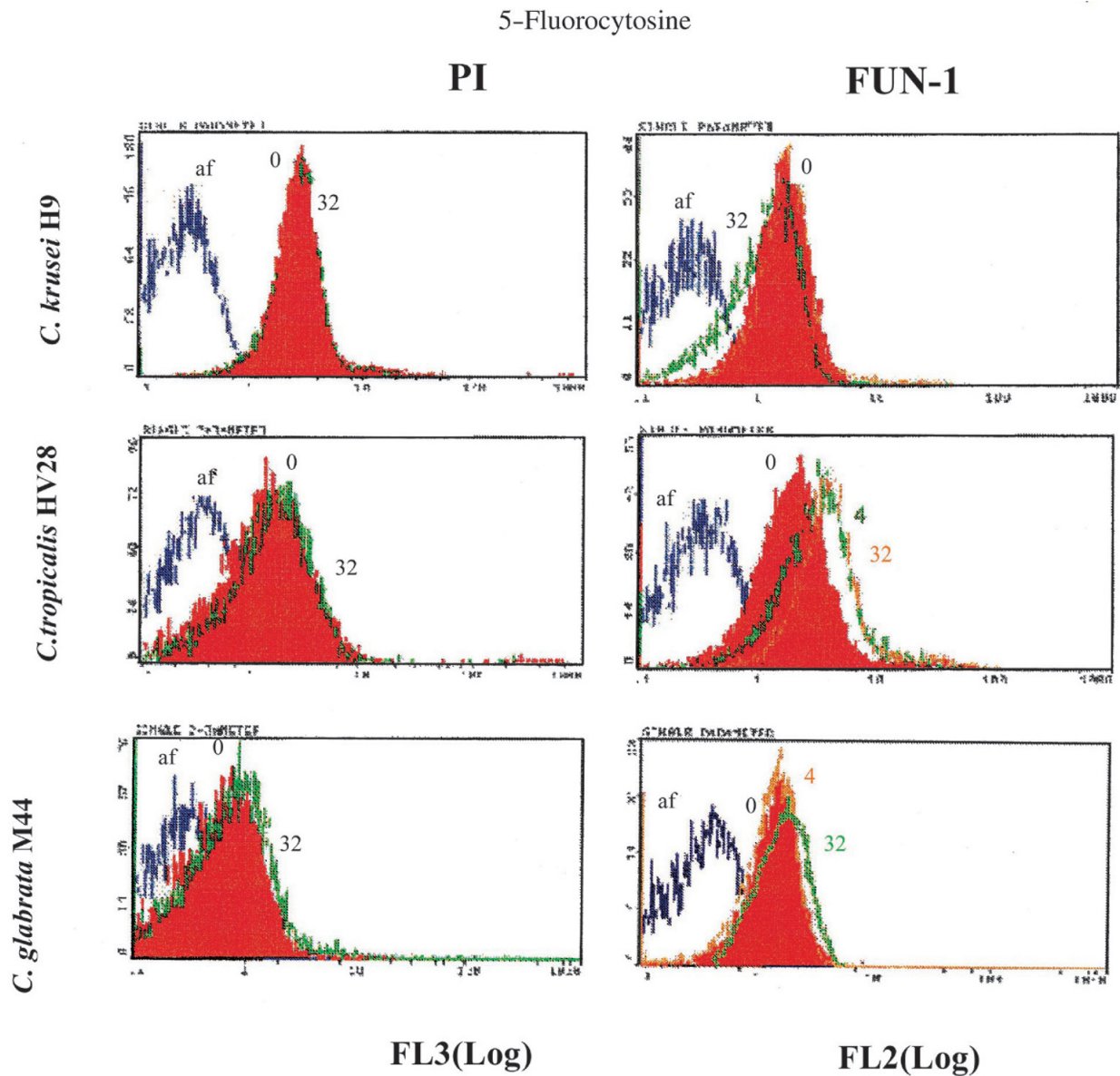
5-Fluorocytosine

Figure 6 Flow cytometry of the strain *Candida krusei* H9 (an example of a strain that is resistant to 5-FC) and *Candida tropicalis* HV28 (an example of a strain susceptible for 5-FC) and *Candida albicans* M44 (an example of an intermediate strain to 5-FC) after treatment with 0.4 and 32 mg/L of 5-FC (0, 4 and 32 on the figure) for 1 h and stained with propidium iodide (PI) or FUN-1. The autofluorescence (af) of the cells is also represented.

DISCUSSION

In this paper three probes were optimised for use in susceptibility testing to three of the most widely used antifungals. FCM was shown to be a good alternative for the study of metabolic or functional alterations in yeast cells. EPM was an excellent tool to help in cytometric interpretation. However, no single probe was useful to study all three drugs. PI is one of the most popular fluorescent probes used in susceptibility testing by cytometric methods [5,19]. However, we found it to be inadequate to evaluate the susceptibility to the tested antifungals. This may be explained by the need for significant damage to the cell membrane to take place to allow the access of PI to the target. According to our previous studies *Candida* cells exposed to drugs that are fungicidal by direct membrane damage, such as ibuprofen [20], benzydamine and local anesthetics [21], incorporated PI in a dose-dependent manner. Also, the activity of echinocandin B could be studied with PI, after 3-h treatment

[22]. Although Am B is a fungicidal drug, inducing K$^+$ leakage [23], it is a large molecule that acts on the yeast cytoplasmic membrane in a way that probably interferes with the uptake of most molecules. Thus, the uptake of PI by Am B-treated cells, compared to untreated cells, is significantly reduced, as the cells become increasingly more impermeable in the presence of Am B. Ramani et al. [19] was able to increase red fluorescence with PI staining on Am B- and Flu-treated cells, but only after association with a membrane detergent (sodium deoxycolate).

Am B-treated cells also remained impermeable to FUN-1, with less staining by FCM than untreated cells, and showing no CIVS under EPM. According to other reports, in yeast cells treated with Am B for 3 h, 4 additional hours were needed for penetration of ethidium bromide into the membrane [18]. A decrease in the uptake of DIOC5 (another fluorescent probe) with increasing concentrations of Am B was also described [24,25]. We used JC-1 to study the susceptibility of yeasts to Am B for the first time. It allowed us to study the effect of Am B, probably due to its lipophilic properties which facilitate the crossing of the membrane and the staining of the yeast cells.

The decreased forward-angle light scatter seen on Am B-treated cells reflects minor morphological changes (decrease in size), already described [18].

Azoles are fungistatic drugs, with no effect on yeast cell viability. Accordingly, we found that after 1-h treatment with Flu, PI could not enter the cell. FUN-1 is an interesting marker with which to study yeast metabolism, which has previously been used to study functional changes induced by acetic acid on yeasts [17]. We found it to be extremely useful to distinguish S (SI > 1) from S-DD (SI < 1 with Flu 8 µg/L and SI > 1 with Flu 64 µg/L) and R strains (SI < 1) to Flu. The decrease in SI by FCM and the corresponding observation under EPM that a large number of cells had CIVS in R strains could be explained by the presence of efflux pumps [26, 27], which are a frequent mechanism of resistance to azoles [27]. Thus, the fluorescent probe might be exported from the yeast cell, together with the antifungal.

Treatment with 5-FC for 1 h did not kill the cells, as determined by CFU counts (see on Table 2) and was unable to make the cells permeable to PI, in accordance with Pore [5] (Figure 6). However, a significant metabolic disturbance was detected with FUN-1 staining, as both S and I strains showed a dose-dependent increase of fluorescence at FL2 (Figure 6) and a decrease in the percentage of cells with CIVS (not shown).

Our results show that JC-1 can also be useful to detect resistance to Flu and to 5-FC, although it was not sensitive enough, under the assayed conditions, to distinguish between S-DD (Flu) or I (5-FC) and S patterns.

According to our data, susceptibility to Am B could be detected after 1 h of exposure to the antifungal and staining with JC-1, susceptibility being correlated with the absence of J-aggregates and a decrease in mean value of FL3 : FL1 fluorescence. Susceptibility to Flu and 5-FC could be determined after incubation with the drugs for 1 h followed by staining with FUN-1. A SI > 1 indicates that the strain is S and a SI < 1 that it is R, having an excellent correlation with the MIC results.

In conclusion, our results demonstrate an excellent correlation between the results of cytometric methods and the classic susceptibility methods for the assayed antifungals. Cytometry allows a significant time-saving in antifungal susceptibility testing compared to classical protocols. This is of particular interest and importance when reliable and accurate answers are needed in the shortest possible time, as is the case in immunocompromised patients, like such as those with leukemia and bone marrow recipients, in whom we cannot afford the chance of mistakes.

ACKNOWLEDGMENTS

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