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Rapid Susceptibility Testing of *Candida albicans* by Flow Cytometry

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The emerging magnitude of human fungal infections has renewed interest in developing rapid and standardized methods for susceptibility testing. We demonstrated that susceptibility testing of *Candida albicans* can be accomplished rapidly by using flow cytometry. Test results were available within 8 to 24 h after *C. albicans* isolates were incubated with amphotericin B, itraconazole, and flucytosine. This is an improvement of 24 to 60 h in the time to availability of susceptibility test results compared to the time to availability of National Committee for Clinical Laboratory Standards-recommended broth macrodilution test results. In addition, the flow cytometric endpoints, mean channel fluorescence, and number of fluorescence-labeled *C. albicans* cells were easy to interpret for greater sensitivity and reliability. Flow cytometry provides a more accurate means of obtaining antifungal susceptibility test results.

The incidence of severe fungal infections has steadily increased during the last five decades with the advent of broad-spectrum antibacterial agents and more aggressive chemotherapies (5, 11). Furthermore, fungal infections have become a major cause of mortality among the growing group of immunocompromised patients, especially those with AIDS (11, 31, 37). Even more disturbing are the frequent reports that fungi are developing resistance to antifungal agents (4, 14, 21, 26, 32, 33, 37, 38). This has facilitated efforts to develop a more reliable in vitro susceptibility test to predict clinical outcome.

The National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Testing has developed guidelines for performing a reproducible method for broth dilution susceptibility testing of yeast (22). The method uses semisynthetic RPMI 1640 medium at pH 7.0 with a spectrophotometrically standardized inoculum and fixed incubation and endpoint criteria (22). The method, however, is labor-intensive, requires incubation for 48 or 72 h, and relies on visual assessment of growth inhibition. More recently, other methods including the Etest (32), the Bioscreen microdilution method (36, 39), and a colorimetric test (8) have been shown to be valuable alternatives to the NCCLS reference method. Collectively, these methods have substantially improved the clinical utility of in vitro susceptibility testing of antifungal agents.

Another approach for performing antifungal susceptibility testing involves the use of flow cytometry (13, 24, 27–30). In this report, we present evidence that flow cytometry can be used to detect rapidly the susceptibility of *Candida albicans* to several antifungal agents including amphotericin B (AMB), flucytosine (5-FC), and itraconazole (ITR). Results were available within 8 to 24 h after the initiation of testing and did not involve complicated steps for avoiding quenching of fluorescence (24) or sustained incubation periods with impermeant or permeant dyes (24, 27). In addition, several parameters of flow cytometric analysis could be used reliably to predict the results of susceptibility testing.

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MATERIALS AND METHODS

Antifungal agents. AMB, 5-FC, and ITR were obtained from Sigma, St. Louis, Mo.; United States Pharmaceutical Company, Inc., Rockville, Md.; and Research Diagnostics, Inc., Flanders, N.J., respectively. A stock solution of AMB was prepared with 100% dimethyl sulfoxide (Sigma) at 4,000 μg/ml, while a stock solution of ITR was prepared in polyethylene glycol 400 (Sigma) at 5,000 μg/ml. Subsequently, the stock solutions were diluted with RPMI 1640 medium containing L-glutamine and 0.165 M morpholinepropanesulfonic acid without sodium bicarbonate (American Biorganics, Inc., North Tonawanda, N.Y.) to concentrations of 1,280 and 1,600 μg/ml AMB and ITR, respectively. The stock solutions were then frozen in 0.5-ml aliquots at −70°C. A stock solution of 5-FC was prepared with sterile distilled water to a concentration of 2,000 μg/ml before being diluted with RPMI 1640 medium to a concentration of 640 μg/ml and frozen in 4.0-ml aliquots at −70°C.

Yeast and yeast preparation. *C. albicans* ATCC 90028 was obtained from the American Type Culture Collection (Rockville, Md.). Other isolates of *C. albicans*, *C.* (*Torulopsis*) *glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. lusitaniae* were obtained from Kenneth V. I. Rolston, M. D. Anderson Cancer Center, Houston, Tex. The strains were grown in 10 ml of brain heart infusion broth (Difco, Detroit, Mich.) at 35°C for approximately 18 h or until the turbidity of the suspensions was equivalent to that of a McFarland no. 1 standard. Each suspension was then diluted twofold with brain heart infusion broth containing 20% glycerol (Sigma), dispensed into 1.5 ml screw-cap tubes (Sarstedt, Newton, N.C.), sealed, and stored at −20°C.

Dyes. Acridine orange (AO), ethidium bromide (EB), and fluorescein diacetate (FDA) were obtained from Sigma. Stock solutions of AO, EB, and FDA were prepared according to the instructions of the manufacturer to yield concentrations of 20, 2, and 5 mg/ml, respectively. The stock solutions were then filter sterilized through a 0.22-μm-pore-size filter apparatus (Arcodisc; Gelman Sciences, Ann Arbor, Mich.) and diluted with phosphate-buffered saline (pH 7.4). The stock solutions were used to prepare fresh concentrations of dyes before each testing procedure. In preliminary studies the dyes were titrated to determine the concentration of each dye that would most effectively stain the yeast. The final concentrations were 8 μg/ml for AO, 4 μg/ml for EB, and 1.8 μg/ml for FDA.

Antifungal susceptibility testing. Broth macrodilution MICs were determined by the NCCLS M27-P methodology (22). Briefly, serial twofold dilutions of the antifungal agents were prepared with RPMI 1640 medium. The final concentrations of the antifungal agents ranged from 12.8 to 0.025 μg/ml for AMB, 64 to 0.125 μg/ml for 5-FC, and 16 to 0.03 μg/ml for ITR. The suspensions containing antifungal agents were then inoculated with 0.5×10^4 to 2.5×10^4 cells per ml and incubated in air at 35°C for 48 h. The MIC endpoint was the lowest concentration of antifungal agent that completely inhibited growth (AMB) or that produced an 80% reduction of turbidity (5-FC and ITR) when compared with that of the drug-free control.

Antifungal susceptibility testing by flow cytometry. Broth macrodilutions were prepared as recommended by NCCLS (22), except that the final inoculum of *C. albicans* was 1×10^5 to 5×10^5 cells per ml. The suspensions containing antifungal agents and *C. albicans* were then incubated at 35°C for 8 and 24 h before analysis by flow cytometry.

Acquisition of flow cytometric data. After incubation of the assay suspensions for 8 and 24 h, 100 μl was removed and diluted with 400 μl of phosphate-buffered saline (pH 7.4), and 50 μl of AO, EB, or FDA was added. The samples were then

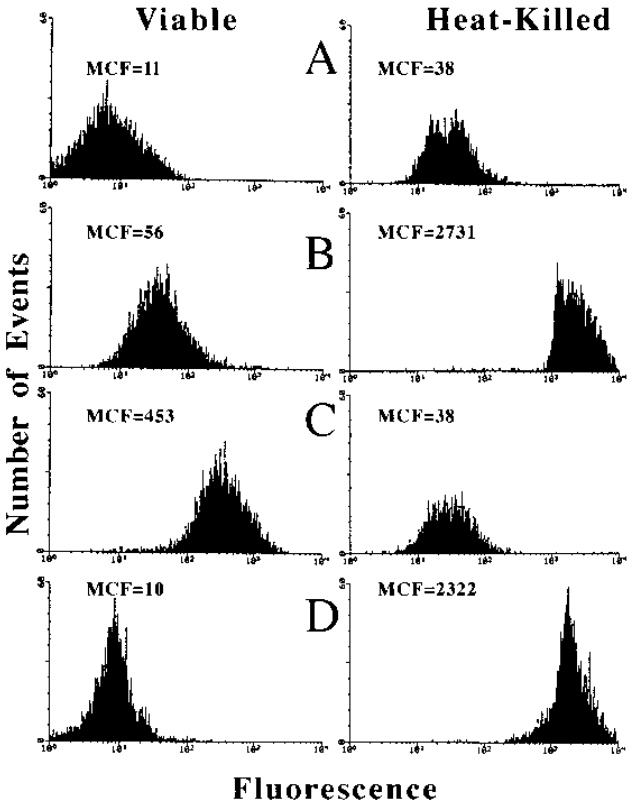

FIG. 1. Histogram profiles of mean channel fluorescence (MCF) values for viable and heat-killed *C. albicans* in RPMI 1640 medium unstained (A) or in the presence of AO (B), FDA (C), or EB (D).

inoculated with each dye at ambient temperature for approximately 5 min before being analyzed with a Bryte HS flow cytometer (Bio-Rad, Hercules, Calif.) with WinBryte software or a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) with FACScan Lysys II software for data acquisition and analysis. Initially, viable and heat-killed *C. albicans* organisms were detected and differentiated in RPMI 1640 medium by using forward scatter, side scatter, and AO, EB, and FDA fluorescence. Live gating was performed on profiles of *C. albicans* during data acquisition to exclude all particles in RPMI 1640 medium. In addition, calibration beads were tested daily, and the mean channel fluorescence did not vary by more than six channels. Controls included samples containing viable, heat-killed, and AMB-killed *C. albicans* in RPMI 1640 medium. Data were acquired for 120 s or until 5,000 counts from gated events were obtained.

Flow cytometric statistical analysis. Samples were analyzed by using the histogram profiles of AO, EB, and FDA fluorescence by using FACScan Lysys II or WinBryte software. Gates were established for viable and heat-killed *C. albicans* on the basis of their reactions with AO, EB, and FDA. Two parameters were evaluated: events per minute (number of labeled *C. albicans* organisms) and mean channel fluorescence (intensity of fluorescence-labeled *C. albicans*). These values were obtained as part of the flow cytometric statistical analysis and were dependent upon the establishment of gates for live and heat-killed *C. albicans*. In addition, samples were analyzed by two-dimensional contour plot profiles of side-angle light scatter versus AO fluorescence by using the flow cytometric softwares. Contour plots were used for their ability to represent populations of *C. albicans* organisms.

Statistics. The values obtained were tested by analysis of variance. The alpha level was set at 0.05 before the experiments were started.

RESULTS

Detection of *C. albicans* by flow cytometry. Initially, viable and heat-killed *C. albicans* organisms were stained with AO, EB, or FDA (Fig. 1). Nonstained viable and heat-killed *C. albicans* organisms were readily detected in RPMI 1640 medium, with mean channel fluorescences of 11 and 38, respectively (Fig. 1A). When viable and heat-killed *C. albicans* organisms were stained with FDA (Fig. 1C), only viable *C. albicans* organisms demonstrated a significant ($P < 0.001$) fluorescence intensity, with a mean channel fluorescence of 453. Both AO (Fig. 1B) and EB (Fig. 1D) stained heat-killed *C. albicans* organisms, with mean channel fluorescences of 2,731 and 2,322, respectively. AO also weakly stained viable *C. albicans*, with a mean channel fluorescence of 56. When these experiments were repeated with other isolates of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. lusitaniae*, similar results were obtained. By contrast, EB failed to stain viable *C. albicans* organisms. All subsequent experiments, however, used AO because it stained both viable and heat-killed *Candida* organisms. The ability of AO to stain weakly isolates of *C. albicans* and other species of *Candida* made initial detection of the yeasts by flow cytometry relatively easy. In addition, maximum separation between viable and heat-killed organisms by using intensity of fluorescence (mean channel fluorescence) was obtained with AO (Fig. 1C). When these experiments were repeated three times, similar results were obtained.

Establishment of gates (regions) for detection of viable and killed *C. albicans*. The purpose of establishing gates was to establish regions R2 and R3 for detection of viable and heat-killed *C. albicans* organisms (Fig. 2). Viable (Fig. 2B) and heat-killed (Fig. 2C) organisms were easily detected within regions R2 and R3, respectively. Region R2 was drawn to contain 95% of the viable *C. albicans* organisms (Fig. 2B). Subsequently, the *C. albicans* organisms were exposed to concentrations of antifungal agents that killed *C. albicans* (no growth on subculture). Approximately 96, 99, and 65% of the organisms were detected in region R3 after exposure to AMB,

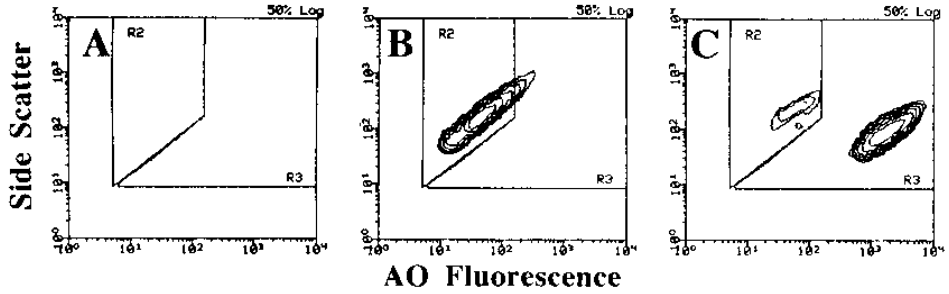

FIG. 2. AO fluorescence versus side-scatter contour plot profiles of RPMI 1640 medium with AO (A), RPMI 1640 medium with AO-labeled viable *C. albicans* (B), and RPMI 1640 medium with AO-labeled heat-killed *C. albicans* (C). The AO-labeled viable *C. albicans* organisms were used to draw the R2 region to include greater than 95% viable cells. AO-labeled heat-killed *C. albicans* organisms were used to draw the R3 region. The R2 and R3 regions were also based upon changes in mean channel fluorescence and side scatter obtained with the AO-labeled viable and AO-labeled heat-killed *C. albicans*. Region R1 was subdivided into regions R2 and R3.

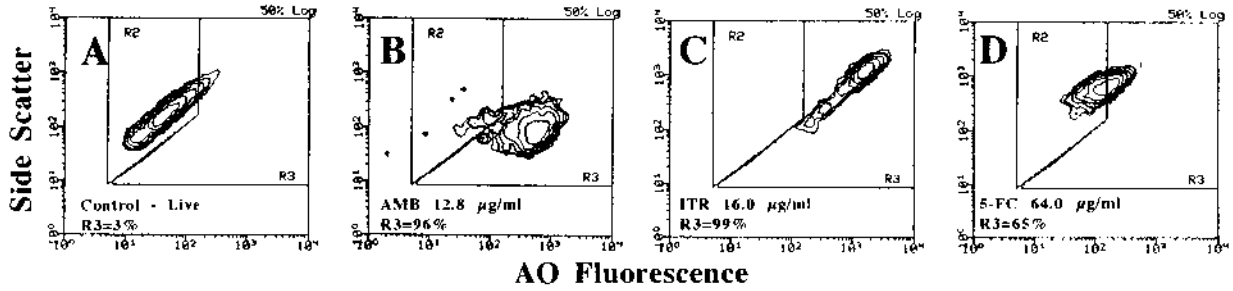

FIG. 3. AO fluorescence versus side-scatter contour plot profiles of viable *C. albicans* (A) and *C. albicans* exposed to concentrations of AMB (B), ITR (C), and 5-FC (D) that killed *C. albicans*. Region R3 contained 96, 99, and 65% *C. albicans* organisms after exposure to AMB, ITR, and 5-FC, respectively. Region R1 was subdivided into regions R2 and R3.

ITR, and 5-FC, respectively (Fig. 3). These results were replicated.

Determination of the susceptibility of *C. albicans* to antifungal agents by flow cytometry. Figure 4 indicates the percentage of AO-labeled *C. albicans* organisms detected in regions R2 and R3 with or without exposure to various concentrations of AMB for 24 h. The percentage of AO-labeled *C. albicans* organisms detected in region R3 increased rapidly after exposure of *C. albicans* to 0.4 μg or more of AMB per ml. Approximately, 13% of the *C. albicans* organisms were detected in region R3 after exposure to 0.4 μg of AMB per ml. Exposure of *C. albicans* to 12.8 μg of AMB per ml resulted in approximately 99% of the organisms being intensively labeled with AO in region R3. Similar results were obtained when the *C. albicans* organisms were exposed to various concentrations of AMB for only 8 h. The MIC of AMB for *C. albicans* determined by the NCCLS broth macrodilution test was 0.8 μg/ml.

We also determined the effects of various concentrations of 5-FC and ITR on the percentage of AO-labeled *C. albicans* detected in region R3 after incubation for 8 and 24 h. The percentage of AO-labeled *C. albicans* increased rapidly from 31 to 87% after exposure to ITR at 0.25 μg/ml and a final concentration of 16 μg of ITR per ml after 8 h of incubation. These results remained consistent even after incubation of *C. albicans* with ITR for 24 h. The MIC of ITR detected by the broth macrodilution method was 0.5 μg/ml. By contrast, the percentage of AO-labeled *C. albicans* organisms detected in region R3 after exposure to 5-FC could not be consistently determined without an incubation period of 24 h. At that time, the proportion of AO-labeled *C. albicans* organisms increased from 24 to 77% after exposure to 0.125 and 64 μg of 5-FC per ml, respectively. The MIC by the broth macrodilution method was 2 μg/ml.

The results presented in Fig. 5 indicate that the percentage of AO-labeled *C. albicans* organisms detected in region R3 correlated inversely with the number of events (*C. albicans*) detected by flow cytometry. In general, the percentage of AO-labeled *C. albicans* organisms detected in region R3 increased rapidly with increasing concentrations of the antifungal agents, while the number of *C. albicans* organisms (events) rapidly decreased. The point of intersection of these curves closely approximated the MICs of AMB, 5-FC, and ITR obtained by the broth macrodilution method. The data in Table 1 indicate that the MICs predicted by flow cytometry were equal to or were within 2 dilutions of the MICs determined by the broth macrodilution method. Most importantly, the flow cytometric MICs were obtained 24 to 32 h sooner than the MICs obtained by the NCCLS broth macrodilution standard test.

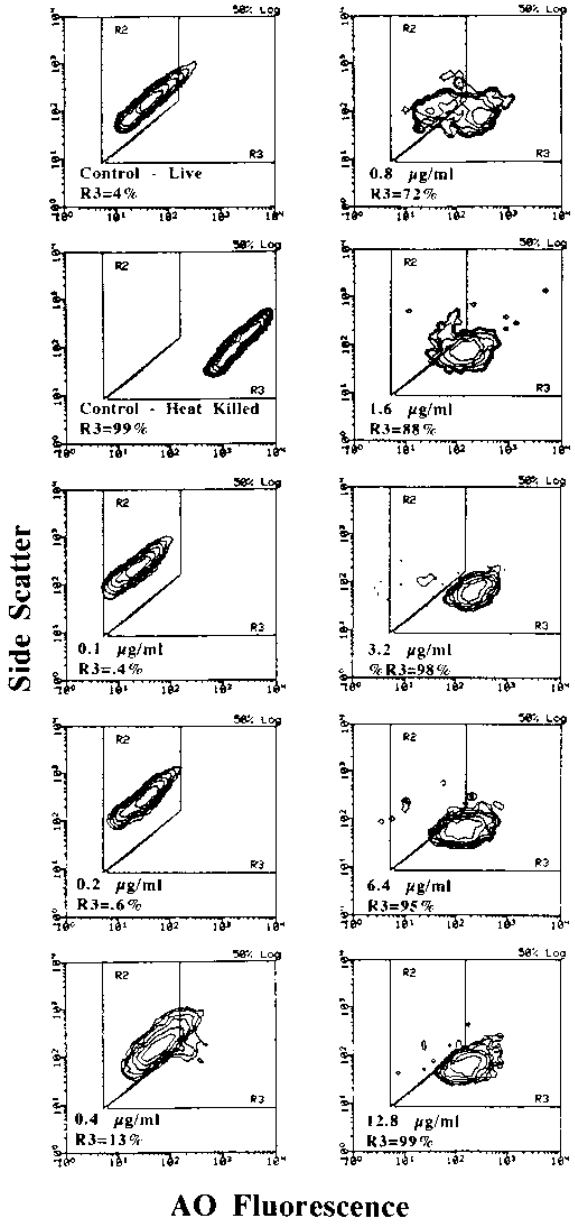

FIG. 4. AO fluorescence versus side scatter contour plot profiles of viable *C. albicans*, heat-killed *C. albicans*, and *C. albicans* exposed to various concentrations of AMB for 24 h. The results indicate the transition of *C. albicans* organisms from the R2 region (viable) to the R3 region (dead) with exposure to increasing concentrations of AMB.

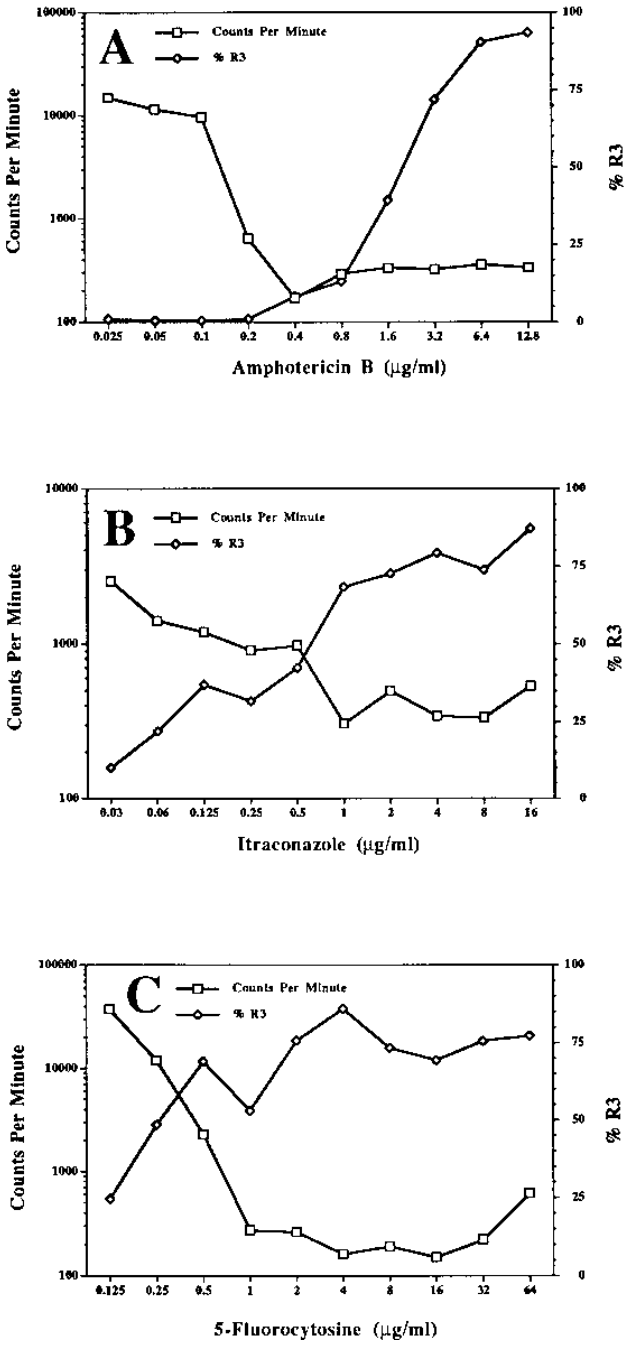

FIG. 5. Effects of various concentrations of AMB (A), ITR (B), and 5-FC (C) on the percentage of *C. albicans* organisms detected in the R3 region and on the number of yeast cells detected per minute by flow cytometry. Results were obtained after 8 h of incubation with AMB and ITR and 24 h with 5-FC. The MICs obtained by flow cytometry were 0.4, 0.5, and 0.5 μg/ml for AMB, ITR, and 5-FC, respectively. These values were obtained by flow cytometry with a 10-fold greater inoculum than that recommended by NCCLS for the standard broth method.

DISCUSSION

A standardized method for performing in vitro susceptibility testing of fungal agents, especially *Candida* spp., has been proposed by NCCLS (22). The proposed method is a broth macrodilution test that has demonstrated good inter- and intralaboratory reproducibilities (9). However, the standardized method is labor-intensive and cumbersome to perform, and endpoints are difficult to determine, especially for antifungal agents other than AMB. Another disadvantage is that incubation periods of 48 to 72 h is required for determination of the in vitro antifungal susceptibility test results. Although standardization is important, investigators (7, 12) have stressed the need for the development of simplified methods to improve the likelihood of use of antifungal susceptibility testing by clinical laboratories.

Recently, there has been a proliferation of reports promulgating the advantages of flow cytometry for performing susceptibility testing of yeast and bacteria (2, 10, 13, 18, 19, 24, 25, 27–30, 34, 35). Flow cytometry simultaneously measures qualitative and quantitative characteristics of individual cells in a heterogeneous population of microorganisms by movement of cells past optical or electronic sensors. Individual cells can be rapidly and precisely detected, counted, characterized, and identified. In addition, flow cytometry has been used to determine the viability of microorganisms by measuring uptake of fluorochrome dyes (6, 10, 13, 15, 16, 18, 20, 24, 25, 34). This latter feature has encouraged evaluations of flow cytometry as an alternate approach to performing susceptibility testing (1, 2, 10, 18, 23–25, 27).

In this report, we showed that susceptibility testing of *C. albicans* could be accomplished rapidly by using flow cytometry. Test results were readily available within 8 h after *C. albicans* organisms were incubated with the antifungal agents AMB and ITR. Furthermore, susceptibility results for 5-FC were obtainable within 24 h after the initiation of testing. This was an improvement of 24 to 32 h in the time to the availability of susceptibility test results compared with the time to availability of those obtained by the NCCLS-recommended broth macrodilution method (22). In addition, the flow cytometric endpoints, mean channel fluorescence (intensity of fluorescence-labeled organisms), and number of events (number of *C. albicans* organisms) were easy to interpret for greater sensitivity and reliability. Frequently, the endpoints obtained by the broth dilution method are difficult to interpret. The assay tubes show partial inhibition of fungal growth or produce a trailing phenomenon, especially with ITR.

These results confirmed and extended the findings of O'Gorman and Hopfer (24). Our assay system, however, did not require steps for removal of antifungal agents. O'Gorman and Hopfer (24) showed that AMB had to be removed from suspensions of *C. albicans* prior to analysis by flow cytometry to prevent quenching of fluorescence-labeled yeast. In our assay system AO was used to detect nonviable yeast, while O'Gorman and Hopfer (24) used EB. This difference in detection dyes may account for our inability to detect quenching of fluorescence-labeled yeast when suspensions of *C. albicans* were exposed to antifungal agents, labeled with AO, and analyzed by flow cytometry. In addition, our assay system did not require long periods of incubation with the detection dye before analysis by flow cytometry. By contrast, O'Gorman and

TABLE 1. MICs of AMB, ITR, and 5-FC for *C. albicans* and duration of incubation for detection by flow cytometry and the NCCLS broth macrodilution test

Antifungal agent	Flow cytometry		NCCLS test	
	MIC (μg/ml)	Time (h)	MIC (μg/ml)	Time (h)
AMB	0.4	8	0.8	48
ITR	0.5	8	0.5	48
5-FC	0.5	24	2.0	48

Hopfer (24) showed that 4 h of incubation with EB was required to label yeast for detection of susceptibility to AMB by flow cytometry. We found that AO weakly labeled viable *C. albicans* and intensively labeled heat-killed and antifungal agent-treated *C. albicans* within minutes (<5 min) of exposure to AO. This finding is important because it also reduces the potential for dye-induced toxic effects that could influence the susceptibility test results (27).

Our results also indicated that the MICs of AMB and ITR obtained by flow cytometry agreed with those obtained by the NCCLS broth macrodilution test. Despite a 10-fold increase in inoculum, the MICs of AMB and ITR obtained by flow cytometry were equal to or within a dilution of those obtained by the NCCLS standard test. By contrast, the MIC of 5-FC for *C. albicans* obtained by flow cytometry was fourfold lower (0.5 μg/ml) than the MIC (2.0 μg/ml) obtained by the broth macrodilution test. In general, the MICs obtained by flow cytometry should be lower than those obtained by conventional methods. The flow cytometer can differentiate within hours viable and nonviable microorganisms exposed to antifungal agents. An operational definition of susceptibility that distinguishes between viable and nonviable yeast by using mean channel fluorescence (percentage shift into region R3) or the number of yeast cells (events) in the suspensions with or without exposure to antifungal agents can readily be established. The broth macrodilution test, however, depends on visualization of growth of *C. albicans* after 48 h of incubation for determination of the MIC. As the concentration of the antifungal agent decreases by deterioration during the 48-h incubation period, the surviving yeast would multiply to produce MIC endpoints higher than those obtained by flow cytometry. The endpoints for each of the antifungal agents now needs to be established with a large number of fresh clinical isolates of *C. albicans* and *Candida* species.

Besides rapid detection time, another advantage of using flow cytometry for the assessment of susceptibility test results is its ability to distinguish between viable and nonviable organisms. We (1, 17, 23) and others (2, 3, 6, 10, 13, 15, 16, 18, 20, 24, 25, 27–30, 34, 35) have shown that uptake of permeant and impermeant dyes by microorganisms correlates with nonviability due to treatment with heat or antimicrobial agents. Although conventional MIC procedures are widely used, they were established for the convenience of interpretation by busy clinical microbiology laboratories. The minimal fungicidal concentration may be a more meaningful and useful measure for assessing resistance or susceptibility results. In addition, larger inocula can be evaluated by flow cytometry, which increases the chances that the assay system can find resistant yeast by detecting large numbers of unstained cells. Technical problems, however, have generally limited usage of the minimal fungicidal concentrations to specialized infectious diseases laboratories. Flow cytometry offers the advantage of determining the minimal fungicidal concentrations within hours of the initiation of testing. It is hoped that these studies will improve the correlation between in vitro fungal susceptibility test results and prediction of clinical outcome.

In conclusion, we have shown that flow cytometry with AO staining of yeast shows considerable promise as a rapid method for obtaining susceptibility test results. The assay can be completed within 8 to 24 h of the initiation of testing.

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