Rapid flow-cytometric susceptibility testing of *Candida* species

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**Objectives**: To develop a rapid flow-cytometric antifungal susceptibility test and to compare results with the standard methods.

**Methods**: Reference and laboratory strains of *Candida* were tested for susceptibility to fluconazole and echinocandin by fluorescent flow cytometry using Acridine Orange as indicator of viability. Flow cytometry results were compared with MICs as determined by macrodilution and/or Etest.

**Results**: Seventy *Candida* strains were tested for susceptibility to fluconazole, and 74 strains for susceptibility to echinocandin. Minimal concentration of fluconazole causing 40% cell damage, as determined by flow cytometry, showed excellent association with MIC, as determined by other methods. The flow method, completed within 5 h, had excellent sensitivity and specificity to distinguish between sensitive, susceptible dose-dependent and resistant strains. The flow cytometry method for echinocandin was completed within 3 h, and minimal concentration causing 50% cell damage was associated with MIC as determined by macrodilution.

**Conclusions**: Antifungal susceptibility testing by FACS is a reliable, rapid method for determining susceptibility of *Candida* to fluconazole and echinocandin. The method allows same-day results, assisting in the selection of appropriate antifungal therapy.

Keywords: fungal, MIC, FACS, fluconazole, echinocandin

**Introduction**

During the past few years, there has been an increasing incidence of invasive fungal infections, especially by *Candida* species other than *Candida albicans*. At our institution, these species currently represent more than half of yeast blood-culture isolates. Although most strains of *C. albicans* are intrinsically sensitive toazole antifungal agents, other *Candida* species have more unpredictable susceptibility patterns and may be dependent upon factors such as patient age and geographic location. In addition, whereas there is controversy as to the relevance of *in vitro* susceptibility results and clinical outcome, recent studies have shown good correlation in certain clinical situations, such as mucosal and invasive candidiasis, especially with non-*Candida Albicans* spp.

The standard method for fungal susceptibility testing for azoles is the broth dilution method proposed by the NCCLS. However, because the method is labour intensive and often presents problems with interpretation, especially the trailing phenomenon observed with azoles, other methods have been explored. The most intensively studied alternative method has been the Etest and good correlation with the NCCLS method has been reported. Despite the good correlation, the broth dilution and Etest methods require 24–48 h for final results, and attempts have been made to develop reliable, more rapid tests. Several years ago, it was suggested that flow cytometry might be useful for susceptibility testing of microorganisms, as drug-induced cell damage could be assessed by use of various fluorescent dyes on a cell-by-cell basis. Some investigators have assessed cell damage by measuring the extent of penetration of vital dyes, by changes in membrane potential as measured by membrane potential-sensitive dyes, or by using dyes sensitive to intracellular metabolic changes. After using various flow cytometry protocols, which proved to be technically difficult, we tested
whether the simple method of Kirk et al.,8 using Acridine Orange (AO), could yield rapid accurate results.

Materials and methods

Test organisms

A total of 62 clinical isolates were tested. These included 31 isolates of C. albicans, 14 of Candida tropicalis, seven of Candida parapsilosis, eight of Candida glabrata, and two of Candida krusei.

Reference strains

Reference strains included C. albicans ATCC 26278, C. albicans ATCC 24433, C. albicans ATCC 90028, C. albicans ATCC 90029, C. tropicalis ATCC 750, C. parapsilosis ATCC 22019, C. krusei ATCC 6528 and a fluconazole-resistant mutant C. albicans, kindly provided by Merck Research Laboratories (USA). In addition, for our echinocandin experiments, four strains of C. albicans were kindly provided by Merck Research Laboratories (USA), and included a laboratory-generated echinocandin-resistant mutant.

Antifungal agents

Fluconazole was provided as stock solution by the manufacturer (Pfizer, Orsay, France). Echinocandin (L774967, MSD) was kindly provided as a pure powder by the manufacturer (Merck Research Laboratories, USA). The powder was dissolved in sterile distilled water and frozen as a stock solution at −20°C.

Standard susceptibility tests

Yeast antifungal susceptibility was tested by either broth macrodilution or by Etest, and some strains by both methods. Broth macrodilution was performed according to NCCLS instructions. Briefly, tubes containing two-fold dilutions of fluconazole, with concentrations in the range 0.25–256 mg/L, and tubes containing two-fold dilutions of echinocandin, with concentrations in the range 0.06–8.0 mg/L, were prepared in RPMI broth. Yeast suspension was added to each of the tubes, which were incubated at 35°C for 48 h. MIC was defined as the lowest concentration demonstrating complete growth inhibition for echinocandin and growth of ≤20% of the control tube for fluconazole.

The Etest diffusion method was performed according to the manufacturer’s instructions (AB Biodisk, Solna, Sweden). Briefly, a yeast suspension adjusted to a turbidity equivalent to that of a 0.5

Figure 1. AO fluorescence (x-axis) versus side scatter (SSC; y-axis) of yeasts. The percentage of damaged yeasts appears in the right upper quadrant (R2). (a) C. albicans (ATCC 90028) live control; (b) C. albicans (ATCC 90028) exposed to fluconazole 0.25 mg/L; (c) C. albicans (ATCC 90028) exposed to fluconazole 0.50 mg/L; (d) C. albicans fluconazole-resistant mutant (#18563, MSD) live control; (e) C. albicans fluconazole-resistant mutant (#18563, MSD) exposed to fluconazole 64 mg/L; (f) C. albicans fluconazole-resistant mutant (#18563, MSD) exposed to fluconazole 128 mg/L.
McFarland standard was inoculated by swab onto casitone agar plates, which were incubated at 35°C. Plates were read at 24 and 48 h and the MIC of fluconazole determined as the point of intersection of zone edge and strip at ~80% growth inhibition.

**Antifungal susceptibility testing by flow cytometry**

The conditions for the assay were those of Kirk et al. with slight modifications. Briefly, yeast strains that had been subcultured on Sabouraud agar were diluted in RPMI 1640 medium (Sigma, St. Louis, MO, USA), with added L-glutamine, to a concentration of 5×10^5 cells per mL for the fluconazole assays. The strains were diluted to the same concentration in yeast peptone dextrose (YPD) (BD Biosciences, Sparks, MD, USA) medium for the echinocandin assays. Two-fold dilutions of fluconazole were prepared (0.125–256 mg/L) in RPMI, and of echinocandin (0.06–8 mg/L) in YPD, and 50 μL of the antifungal drug solutions were inoculated with 50 μL of the yeast suspension. The test tubes were incubated at 35°C in a continuously shaking water bath at 200 rpm for 5 h for fluconazole and for 3 h for echinocandin. Following incubation, 400 μL of phosphate-buffered saline (pH 7.4) and 50 μL of AO (Sigma, St Louis, MO, USA), final concentration, 11 mg/L, were added. After vortexing, samples were incubated at room temperature for ~5 min before analysis by flow cytometry (FACSScan Flow Cytometer; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), using an argon laser emitting at 488 nm. Tubes with no antifungal drug served as the viable controls and similar tubes, heat-killed at 100°C for 10 min, at the end of the incubation, served as non-viable controls.

**Acquisition of flow cytometric data**

A gate that excluded debris and cell clusters was adjusted from a cytogram, derived from a forward-scatter versus side-scatter plot. A total of 5000 yeast cells were analysed. Data were analysed by CellQuest software (Becton Dickinson, California, USA) by quadrant statistical analysis of side-scatter versus FL3. Yeast moving into the red fluorescence region (R2) represented damaged cells. Five percent yeasts in this region were allowed as background in the viable control. The percentage of damaged yeasts—as related to drug concentration—was recorded, and correlated to MIC, as determined by standard methods. As the flow cytometry assay measures cell damage rather than inhibition, we prefer to relate to 'minimal damaging concentration' (MDC) rather than MIC.

**Results**

Figure 1 shows a sample dot plot analysis of a fluconazole-susceptible strain, showing >50% damaged yeasts exposed to 0.5 mg/L of fluconazole, and of a fluconazole-resistant strain, showing only 40% damaged yeasts even at a fluconazole concentration of 128 mg/L. Fluconazole concentrations resulting in 30%, 40% and 50% damage are shown for the different Candida strains and compared with MIC (Table 1). All strains of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* were sensitive to fluconazole (MIC ≤ 8 mg/L), and showed a 40% MDC range of 0.25–4 mg/L. Although *C. albicans* had a low 50% MDC (range: 0.25–2 mg/L), *C. tropicalis* and *C. parapsilosis* had a higher 50% MDC (range: 1–16 mg/L and 2–32 mg/L, respectively). *C. glabrata* would be classified as susceptible dose-dependent to fluconazole (MIC 16–32 mg/L) and the 40% MDC range was 2–16 mg/L. The fluconazole-resistant *C. albicans* and strains of *C. krusei* showed 40% MDC of ≥32 mg/L. Thus, 40% MDC could distinguish between fluconazole-sensitive, susceptible dose-dependent and resistant Candida strains, as defined by MICs determined by standard methods.

All Candida strains, except the echinocandin-resistant mutant strain, had MICs of ≤2 mg/L when tested for susceptibility to echinocandin by macrodilution. By flow cytometry, all strains of *C. albicans* demonstrated 50% MDC of ≤0.125 mg/L, and strains of *C. parapsilosis* and *C. krusei* demonstrated 50% MDC of ≤1 mg/L. Only the echinocandin-resistant *C. albicans* strain (MIC > 16 mg/L) demonstrated 50% MDC of 2–4 mg/L. Thus, 50% MDC could rapidly test for echinocandin susceptibility in different Candida strains. This needs to be validated by testing a greater number of resistant strains.

**Discussion**

During the past few years, several reports have been published using flow cytometry methodology for fungal-susceptibility testing. Despite reported successes in shortening the incubation times for acquisition of results, as compared with macrodilution and agar diffusion, several methods were problematic in our hands. Methods using propidium iodide required the post-incubation addition of sodium deoxycholate in order to enhance

<table>
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<tr>
<th><em>Candida</em> spp. (no.)</th>
<th>MIC (mg/L) (range)</th>
<th>MDC (mg/L) (range)</th>
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<tr>
<td></td>
<td>Etest</td>
<td>macrodilution</td>
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<td></td>
<td></td>
<td>30% damage</td>
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<tr>
<td><em>C. albicans</em> (35)</td>
<td>0.75–2</td>
<td>0.5–2</td>
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<tr>
<td><em>C. albicans</em> mutant (1)</td>
<td>256</td>
<td>128</td>
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<tr>
<td><em>C. tropicalis</em> (1)</td>
<td>1.0–6</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (8)</td>
<td>0.75–3</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. glabrata</em> (8)</td>
<td>1–16</td>
<td>1–16</td>
</tr>
<tr>
<td><em>C. krusei</em> (3)</td>
<td>256</td>
<td>32–64</td>
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* Included 31 clinical isolates and one each of the following reference strains: ATCC 26278, ATCC 24433, ATCC 90028, ATCC 90029.
* Included 14 clinical isolates and ATCC 750.
* Included seven clinical isolates and ATCC 22019.
* Included two clinical isolates and ATCC 6528.
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permeability to propidium iodide. The addition of deoxycholate resulted in gelling of the yeast suspension, often during the reading of the sample in the flow cytometer. We could not overcome this problem despite many attempted modifications.

Our attempt to use FUN-1 (Molecular Probes, Eugene, OR, USA) was also unsuccessful. This dye is converted into a red fluorescent probe in actively respiring cells, sequestering in vacuole structures. In non-respiring (dead) cells no accumulation of dye occurs in vacuoles, and the shift to red fluorescence should not be observed. Although we could easily distinguish live from dead cells by fluorescent microscopy, we could not distinguish them by flow cytometry, despite many attempted modifications. The small orange–red vacuoles found in live cells did not produce a stronger red signal than the overflow into the region of red fluorescence by the green fluorescing dead cells.

We next attempted to use the method of Kirk et al. to test susceptibility to fluconazole and echinocandin, drugs that these authors had not tested. We also tested the feasibility of shortening the assay time to 5 h rather than the 8–24 h, as reported by Kirk et al. A 5 h incubation would realistically allow for results to be obtained on the same working day. In our hands, this method was very successful and we were able to complete the fluconazole assay in 5 h and the echinocandin assay in 3 h, allowing same-day results.

An additional aspect of the present study was the correlation between MIC results obtained by macrodilution and/or agar diffusion and flow cytometry results. All previous studies defined the parameter of MIC by flow cytometry, as the drug concentration producing 50% cell damage, measured by percent cells entering the dead cell region. We feel that the term MIC—minimal inhibitory concentration—is inappropriate, as inhibition is not being measured by flow cytometry. A more appropriate term would be MDC, ‘minimal damaging concentration’, and this is the parameter we chose to compare with conventional MIC. The present study demonstrated that 50% MDC might not be the best parameter for predicting the degree of fungal susceptibility to fluconazole under our test conditions. In fact, 30% and 40% MDC were more predictive of sensitive, susceptible dose-dependent and resistant levels of susceptibility of different Candida spp. The effect of echinocandin (L774967, MSD) was very dramatic and although we had only one resistant strain to test, repeated experiments indicated that ≥50% MDC could distinguish sensitive from resistant strains. The ability to obtain results for echinocandin susceptibility in such a short time, 2–4 h, is probably due to the mechanism of its action— inhibition of fungal cell wall synthesis—resulting in rapid cellular damage.

In conclusion, we report a rapid flow cytometry assay for fluconazole and echinocandin fungal susceptibility testing, using AO. Results were obtained in 5 h or less, and compared very well with standard MIC determinations. The method is easy and reproducible and can be implemented in any laboratory with access to a flow cytometer.

Acknowledgements

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References