Novel Fluorescent Broth Microdilution Method for Fluconazole Susceptibility Testing of Candida albicans

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Received 13 October 2000/Returned for modification 5 March 2001/Accepted 19 April 2001

A comparative evaluation of the reference National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method with a novel fluorescent carboxyfluorescein diacetate (CFDA)-modified microdilution method for the susceptibility testing of fluconazole was conducted with 68 Candida strains, including 53 Candida albicans, 5 Candida tropicalis, 5 Candida glabrata, and 5 Candida parapsilosis strains. We found trailing endpoints and discordant fluconazole MICs of ≤8 μg/ml at 24 h and of ≥64 μg/ml at 48 h for 12 of the C. albicans strains. These strains satisfy the definition of the low-high MIC phenotype. All 12 low-high phenotype strains were correctly shown to be susceptible at 48 h with the CFDA-modified microdilution method. For the 41 non-low-high phenotype C. albicans strains, the CFDA-modified microdilution method yielded 97.6% (40 of 41 strains) agreement within ±1 dilution at 24 h compared with the reference method and 92.7% (38 of 41 strains) agreement within ±1 dilution at 48 h compared with the reference method. The five strains each from C. tropicalis, C. glabrata, and C. parapsilosis that were tested showed 100% agreement within ±2 dilutions for the two methods being evaluated.

Candida is the fourth most common cause of nosocomial bloodstream infection in the United States (2, 4), and the rate of primary bloodstream infections continue to increase (3, 9). In the Americas, Candida albicans is the species most frequently isolated from the bloodstream (16). Candidemia is often difficult to diagnose and refractory to therapy. The attributable mortality rate is 38% in the United States (28) and between 19 and 23% in Canada (9, 25, 29).

Use of the broad-spectrum antifungal fluconazole for the treatment of serious systemic Candida infections has increased. Fluconazole is a less toxic alternative to amphotericin B and has been recommended as primary therapy for candidemia in nonneutropenic and stable neutropenic patients who have not received prior fluconazole treatment and in whom Candida krusei is unlikely (5, 24). In vitro susceptibility testing for fluconazole is of clinical importance, as therapeutic success depends substantially on achieving plasma fluconazole levels that are sufficiently higher than MICs (22).

Despite great advances in the standardization of antifungal susceptibility testing, azole endpoint determination continues to be problematic and subjective and a major source of interlaboratory variability (6, 18, 23). The trailing-growth phenomenon is often responsible for these difficulties, whereby partial inhibition of fungal growth occurs over the range of azole concentrations (6, 13, 14, 23).

Previous work has demonstrated the utility of using fluorescent dyes to assess the viability of C. albicans exposed to amphotericin B (10). We have investigated the use of the vitality-specific dye carboxyfluorescein diacetate (CFDA) in the standardized NCCLS M27-A broth microdilution method (12) to assess the susceptibility of Candida spp. to fluconazole. In this study we compared the NCCLS microdilution method with a CFDA modification in determining fluconazole susceptibility for common clinical yeast isolates (C. albicans, Candida tropicalis, Candida glabrata, and Candida parapsilosis).

Antifungal drug. Fluconazole powder (Pfizer-Roerig, Inc.) was dissolved in sterile distilled water to make a stock concentration of 10,000 μg/ml and frozen at −70°C. The stock solution was thawed once, and fresh dilutions were used.

Yeast isolates. Yeast isolates were obtained from the National Centre for Mycology, Division of Microbiology and Public Health, Edmonton, Alberta, Canada, and two low-high phenotype C. albicans strains were kindly supplied by John H. Rex from the Center for the Study of Emerging Pathogens and Reemerging Pathogens, Laboratory of Mycology Research, University of Texas Medical School, Houston, Tex. The identity of the isolates was confirmed by standard methods (27); isolates were stored in skim milk at −70°C and were then subcultured twice on Sabouraud dextrose agar (Difco, Sparks, Md.) before use. These strains included 48 clinical isolates of C. albicans and homologous control strains ATCC 90028, ATCC 90029, ATCC 24433, ATCC 10231, and ATCC 20408; 4 clinical isolates of C. tropicalis and ATCC 750; 4 clinical isolates of C. glabrata and ATCC 90030; and 4 clinical isolates of C. parapsilosis and ATCC 22019.

Antifungal susceptibility testing. The reference NCCLS broth microdilution method was performed as described in the M27-A document (12). Fluconazole concentrations were diluted in RPMI 1640 medium with l-glutamine, Morpholinepropanesulfonic acid (MOPS) buffer at 165 mM and pH 7 (Angus Buffers & Biochemicals, Niagara Falls, N.Y.) and 100-μl aliquots were placed into the wells of 96-well microtiter Linbro plates (Flow Laboratories Inc., McLean, Va.) with
clear, U-shaped well bottoms. The final concentrations of fluconazole ranged from 0 to 64 μg/ml. Six *C. albicans* strains were tested per 96-well plate, which allowed for the empty outermost wells to be filled with sterile water to minimize evaporation.

Five *C. albicans* colonies with a diameter of ≥1 mm were suspended in sterile 0.85% saline and adjusted to a final concentration (after inoculation) of 0.5 × 10^3 to 2.5 × 10^3 cells per ml in RPMI 1640-MOPS medium. The inoculum was added to the fluconazole trays, incubated at 35°C, and evaluated at 24 and 48 h.

(i) **Reference MIC endpoint.** The reference broth microdilution was scored by comparing the growth in each well with that in the growth control (drug-free) well. The MIC was defined as the minimum drug concentration at which visual growth was determined to be 80% relative to that of the growth control.

(ii) **Fluorescent MIC endpoint.** The CFDA-modified microdilution method was identical to the method described for broth microdilution susceptibility testing, except that after determination of visual endpoints at 24 and 48 h of incubation at 35°C, a fluorescence assay was also performed. The supernatants were first removed from the tray wells by using a multichannel pipettor, and the remaining yeasts were resuspended to 200 μl per well in 35°C-warmed 0.1 M MOPS buffer at pH 3.5 with 50 mM citric acid. Into each well, 5 μl of a stock of 5 mg of 5(6)-CFDA (Molecular Probes, Eugene, Ore.)/ml in dimethyl sulfoxide was added for a final concentration of 122 μg/ml. 5(6)-CFDA is a lipophilic, nonpolar substrate that traverses the cell membrane and is hydrolyzed by nonspecific intracellular esterases to the fluorescent anion carboxyfluorescein (10). A multichannel pipettor was used to resuspend well contents by pipetting, alternately filling and emptying the wells 20 times. The tray contents were then incubated in the dark at 35°C for 1 h. The well contents were resuspended again as before, and the trays were assayed for fluorescence with an FL500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, Vt.). 5(6)-CFDA was evaluated using excitation and emission wavelengths of 485 and 530 nm, respectively. The fluorescence of the drug-free control was defined as 100%, and the fluorescence of the fluconazole-exposed wells was reported proportionally to this value (see Fig. 2). The MIC endpoint was defined as the lowest fluconazole concentration at which the fluorescence was reduced to 80% of that of the drug-free growth control. The CFDA microdilution method was performed in triplicate for each yeast strain assayed.

The technical time required to include the CFDA modification to the standard microdilution susceptibility test is primarily dependent on the 1-h incubation time and on the time required using a multichannel pipettor to add the CFDA and resuspend the yeasts in the wells.

The comparative evaluation of the reference NCCLS broth microdilution method and the CFDA-modified microdilution method for susceptibility to fluconazole was conducted with 68 *Candida* strains, including 53 *C. albicans*, 5 *C. tropicalis*, 5 *C. glabrata*, and 5 *C. parapsilosis* strains. The *C. albicans* isolates chosen covered a broad range of susceptibility to fluconazole (Fig. 1). The fluconazole MICs for control strains were within accepted limits, providing an internal control for the NCCLS reference method. Evaluation by the reference microdilution method determined that of the *Candida* strains tested; 12 strains of *C. albicans* manifested extreme trailing endpoints producing discordant MICs at 24 h (<8 μg/ml) and 48 h (≥64 μg/ml). They were thus considered to have the low-high MIC phenotype (20). All *C. albicans* strains with the low-high phenotype tested by the CFDA-modified microdilution method were not discordant between 24 and 48 h. These *C. albicans* strains were all shown to be susceptible at both 24 and 48 h. In fact, the MICs were identical for 11 of the 12 strains.

The CFDA-modified microdilution method allows for the quantification of fluconazole inhibition and the production of dose-response curves (Fig. 2). MICs were determined from these dose-response curves after plotting the 80% inhibition and rounding up to the standardized M27-A endpoint.

Table 1 summarizes the distributions of the differences in MICs and the percent agreement using the reference and CFDA-modified broth microdilution methods for non-low-high phenotype strains of *C. albicans*. Considering the non-low-high phenotype *C. albicans* strains only, the CFDA-modified broth microdilution method yielded 97.6% agreement within ±1 dilution compared with the NCCLS reference method. At 48 h the two methods yielded 92.7% (38 of 41) agreement within ±1 dilution and 97.6% (40 of 41) agreement within ±2 dilutions. Where MIC endpoints differed between the two methods, the interpretive susceptibility category changed for only one strain. The most common discrepancies between the different susceptibilities of *C. albicans* strains to fluconazole were due to the CFDA-modified microdilution MICs being 1 dilution lower than those obtained by the standard method. The five strains each of *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* showed 100% agreement within ±2 dilutions for the two methods being evaluated.

Endpoint determination for fluconazole susceptibility testing is recognized to be problematic and a significant source of interlaboratory variability (6, 18, 23). The trailing-growth phenomenon, which describes the partial inhibition of fungal growth over most or all of the concentration range (6, 13, 14,
FIG. 2. Effect of fluconazole on representative strains of *C. albicans*, which are low-high phenotype (707-15), susceptible (Y91), susceptible-dose dependent (Y98), and resistant (965). Each isolate was tested for susceptibility with the M27-A microdilution method (MIC\textsubscript{M27-A}) and the CFDA-modified microdilution method (MIC\textsubscript{CFDA}) at both 24 and 48 h. Results for the CFDA-modified method are shown graphically as relative fluorescence units. The fluorescence of the growth in the drug-free control was defined as 100%, and the fluconazole-exposed wells were scaled to this value. Error bars indicate standard error. The results of the reference M27-A method are shown below as a digital image of the unagitated growth in the 96-well plate at 24 and 48 h.
Table 1. Distribution of difference between the MICs of fluconazole for 41 strains of C. albicans determined by comparison of the CFDA-modified and reference NCCLS M27-A broth microdilution methods

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>% Discrepancies between methodsa</th>
<th>% Agreement after no. of dilution</th>
</tr>
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<tbody>
<tr>
<td>24</td>
<td>-2  0  -1  0  1  2  &gt;2</td>
<td>1  2</td>
</tr>
<tr>
<td>48</td>
<td>2.4 0  31.7 63.4 2.4 0  0</td>
<td>97.6 97.6</td>
</tr>
</tbody>
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a Includes only strains of C. albicans which do not have the low-high phenotype.
b The numbers −2 to 2 indicate the fold dilution difference (log₂).

23), is largely responsible for the difficulties attributed to endpoint determination, especially with Candida spp. (14). The impediment that this so-called trailing endpoint represents forazole susceptibility testing can with some isolates be exacerbated over time, producing discordant MICs of <8 μg/ml at 24 h and of ≥64 μg/ml at 48 h (1, 11, 20, 21). Such discordant MICs place these isolates into susceptible and resistant MIC interpretive categories at 24 and 48 h, respectively, if one uses the NCCLS M27-A guidelines (12) and are consequently referred to as having a low-high MIC phenotype (20). The present evidence suggests that the lower MIC obtained at 24 h using the reference microdilution method correlates most closely with the in vivo outcome (11, 20, 21).

The M27-A method establishes the endpoint for susceptibility testing of Candida spp. to azoles at 48 h with a criterion of 80% reduction in growth (12). Modifications of the M27-A reference method are acceptable and expected (6, 21), and it has been suggested that correction can be made for trailing MIC endpoints by shortening the incubation time to 24 h and lowering the MIC endpoint to the lowest drug concentration producing a 50% reduction in growth (15, 21, 22). The requirement of a 48-h incubation for optimal testing conditions may represent a barrier to this change (7, 21). In one study (17) the results for three microdilution susceptibility test formats were shown to be reproducible and in agreement with one another after 24 h but required 48 h to achieve acceptable agreement with the microdilution reference MICs.

Additional studies have proposed the use of a colorimetric endpoint in a microdilution format by including an oxidation-reduction indicator with the yeast and drug prior to incubation (15). However, the colorimetric method also presents trailing azole endpoints at 48 h (15, 26), and some species-specific discrepancies have been observed (15).

The CFDA-modified microdilution method does not alter the M27-A microdilution protocol but instead can be employed at 24 or 48 h to clarify MIC endpoints. The fluorescent dye CFDA is applied postincubation to the microdilution tray and thus does not interfere with the complex interaction between the yeast and antifungal drug.

The fluorescent dye CFDA is a lipophilic, nonpolar substrate that diffuses across the cell membrane and is hydrolyzed by nonspecific intracellular esterases to the fluorescent anion carboxyfluorescein (19). Cells with compromised membranes rapidly leak carboxyfluorescein, even when residual esterase activity is retained intracellularly (8). The utility of CFDA for assessing the vitality of C. albicans exposed to amphotericin B has been previously demonstrated (10).

The CFDA-modified microdilution method allowed for the stringent 80% growth inhibition endpoint to be maintained with fluconazole susceptibility testing. Furthermore, this method permitted the evaluation of MICs at 24 or 48 h with clearly demarcated endpoints despite the trailing-growth phenomenon. The CFDA-modified microdilution method determined that all 12 low-high phenotype strains of C. albicans were susceptible to fluconazole and had identical endpoints at 24 and 48 h. This result supports the in vivo evidence suggesting that the strains of C. albicans with the low-high phenotype are actually susceptible to fluconazole (1, 20, 21). In fact, one of the low-high phenotype strains, 707-15, demonstrated to be susceptible with the CFDA-modified microdilution method, has previously been shown to be susceptible in vivo (21).

There was excellent agreement between the NCCLS M27-A broth microdilution method and the CFDA-modified microdilution method using an 80% inhibition-of-growth endpoint for strains of C. albicans without the low-high phenotype. These results demonstrate that the CFDA-modified microdilution method for testing fluconazole is entirely comparable to the NCCLS reference microdilution method. The one strain of C. albicans for which the fluconazole MIC differed in the two methods being compared was shown to be very resistant using the M27-A microdilution method and susceptible using the CFDA-modified method. This strain has unusual fluorescent staining properties, and investigations are under way to determine the nature of its resistance mechanism.

A small survey of the other three major bloodstream fungal pathogens (16) (C. tropicalis, C. glabrata, and C. parapsilosis) showed excellent agreement between the M27-A microdilution and CFDA-modified microdilution methods. Further studies to evaluate the applicability of the CFDA-modified method with other antifungal agents and other species of yeast are ongoing.

In summary, the CFDA-modified microdilution method provides objective and quantifiable endpoints for fluconazole susceptibility testing at 24 and 48 h which are reproducible and easy to interpret. It eliminates the ambiguity of the low-high phenotype while maintaining the integrity of the NCCLS protocol. This method is simple to perform and provides the opportunity for automation and widespread use.

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