Effects of Incubation Temperature, Inoculum Size, and Medium on Agreement of Macro- and Microdilution Broth Susceptibility Test Results for Yeasts

REBECCA A. COOK, KYLE A. MCINTYRE, AND JOHN N. GALGIANI*

Medical and Research Services, Veterans Administration Medical Center, Tucson, Arizona 85723, and Department of Medicine, University of Arizona College of Medicine, Tucson, Arizona 85724

Received 3 April 1990/Accepted 7 June 1990

We examined the effects of temperature and inoculum on the agreement of macro- and microdilution broth MICs of five antifungal agents against six isolates of Candida species or Torulopsis glabrata. Incubation temperature affected results with amphotericin B, flucytosine, fluconazole, and SCH 39304, producing better agreement at 35°C than at 37°C. Agreement between methods was better with an inoculum size of 10^6 than with one of 10^5 yeast cells per ml in testing fluconazole or SCH 39304, and the discrepancies seen with a higher incubation temperature and a larger inoculum appeared to be additive. However, inoculum size did not seem to affect agreement between methods in testing amphotericin B, flucytosine, or ketoconazole. Regardless of test conditions, macrodilution broth MICs of amphotericin B for different isolates were strikingly higher than microdilution test MICs, with mean differences being greater than ninefold under some test conditions. We conclude that for most currently available antifungal agents, an incubation temperature of 35°C and a starting yeast inoculum of less than 10^4 cells per ml improve the agreement between macro- and microdilution broth tests.

The increasing incidence of fungal infections and the more frequent use of antifungal agents have strengthened the need for useful and reliable antifungal susceptibility test methods (4). In anticipation of this need, collaborative efforts coordinated by the National Committee for Clinical Laboratory Standards have produced new information that may lead to a rational selection of a macrodilution broth method (3, 5, 11) to serve as a reference standard. However, other types of testing procedures may be more practical, and in this context, the correlation of their results with those from macrodilution tests is of interest.

One alternative procedure closely related to that of the macrodilution broth method is the utilization of smaller volumes in microdilution trays (10), which can be more automated and are routinely in use at many clinical laboratories for other testing procedures. In recent studies, we examined the relationship of endpoint results obtained by macro- and microdilution broth methods with several newer antifungal agents (8, 9). Whereas excellent agreement was obtained between results from the two methods for colifungin, significant systematic differences were observed with the new triazoles fluconazole and SCH 39304. Subsequent exploratory studies suggested that these discrepancies could have been influenced by changes in the incubation temperature and yeast inoculum size. For this reason, we examined the effect of these variables on several currently available antifungal agents.

MATERIALS AND METHODS

Yeasts. The following six isolates were used: Candida albicans C17 and ATCC 64546, Torulopsis glabrata 60101, Candida tropicalis F26, Candida parapsilosis 3288, and Candida lusitaniae ATCC 64125. Isolates were stored at −70°C in yeast nitrogen broth containing 10% glycerol, thawed, and maintained on Sabouraud dextrose–3% agar plates (Sabouraud dextrose broth [BLB Microbiology Systems, Cockeysville, Md.] and Bacto-Agar [Difco Laboratories, Detroit, Mich.]). For studies, subcultures were inoculated onto Sabouraud dextrose–3% agar plates and incubated at 37°C overnight.

Drugs. SCH 39304 (Scherering-Plough Research Corp., Bloomfield, N.J.), fluconazole (Pfizer Research, Groton, Conn.), amphotericin B (E. R. Squibb & Sons, Princeton, N.J.), and ketoconazole (Janssen Pharmaceutical Inc., Piscataway, N.J.) were used to prepare 1-mg/ml stock solutions in 2% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) for the triazoles and 10% dimethyl sulfoxide for the other drugs. Stock solutions of flucytosine (Hoffmann-La Roche Inc., Nutley, N.J.) were prepared at a concentration of 10 mg/ml in 10% dimethyl sulfoxide. All drugs were stored at −70°C.

Media and buffers. Unbuffered synthetic amino acid media for fungi (SAAMF) were prepared as previously described (2, 6), except that solutions of amino acids (Irvine Scientific Sales Co., Inc., Santa Ana, Calif.) were used instead of powdered amino acids and cystine was replaced with equimolar amounts of cysteine (7). Yeast nitrogen broth (Difco), RPMI 1640 (Sigma), and high-resolution broth (kindly supplied by Peter Troke, Pfizer Inc., Sandwich, England) were prepared as specified by the manufacturers, sterilized by filtration, and stored at 4°C. On the day of use, morpholinepropanesulfonic acid (MOPS) buffer (Sigma) was added to each medium to a final concentration of 0.165 M, the pH was adjusted to 7.0 with 10 M sodium hydroxide or 6 M hydrochloric acid, and the medium was resterilized by filtration.

Susceptibility testing. Macrodilution broth assays were performed as previously described (8, 9). Briefly, twofold dilutions of the antifungal agents were prepared by standard methods (10) in media to 10 times the final concentrations, as listed below. Yeast inocula were prepared by suspending

* Corresponding author.
TABLE 1. Differences between macro- and microdilution broth MICs for SCH 39304 and fluconazole at two incubation temperatures, with two inoculum sizes, and in four synthetic mediaa

<table>
<thead>
<tr>
<th>Medium</th>
<th>SH 39304</th>
<th>Fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35°C</td>
<td>37°C</td>
</tr>
<tr>
<td></td>
<td>10^2 CFU/ml</td>
<td>10^4 CFU/ml</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>0.40 ± 0.98</td>
<td>4.00 ± 1.10</td>
</tr>
<tr>
<td>HR</td>
<td>0.40 ± 0.75</td>
<td>5.20 ± 2.73</td>
</tr>
<tr>
<td>SAAMF</td>
<td>-0.20 ± 0.80</td>
<td>-0.40 ± 0.75</td>
</tr>
<tr>
<td>YNB</td>
<td>-2.00 ± 0.41</td>
<td>2.20 ± 0.93</td>
</tr>
<tr>
<td>All media</td>
<td>-1.0 ± 0.66</td>
<td>2.00 ± 1.60</td>
</tr>
</tbody>
</table>

a Five yeast isolates were used for each test condition. Negative values denote microdilution MICs larger than macrodilution MICs. HR, High-resolution broth; YNB, yeast nitrogen broth.

several colonies in sterile water and matching the percent transmittance to that obtained with a 0.5 McFarland turbidity standard (11). The suspensions were diluted 1:100 and 1:10,000 in media for use. These dilutions produced final starting inocula for the various isolates ranging from 1.7 × 10^4 to 5.8 × 10^4 CFU/ml (large inocula) and from 1.5 × 10^4 to 9.7 × 10^4 CFU/ml (small inocula), as judged by quantitative subcultures of final suspensions. Nine parts inoculum were combined with one part drug to a final volume of 1.0 ml in optical-quality polystyrene tubes (12 by 75 mm; Falcon, Lincoln Park, N.J.). The resulting drug concentrations were 0.0039 to 512 μg/ml for fluconazole and SCH 39304 and 0.0039 to 8 μg/ml for amphotericin B, fluocytosine, and ketoconazole. The incubation period was 22 to 24 h, except for the C. parapsilosis isolate, which required 46 to 48 h to produce visually detectable growth in the drug-free control tube. Temperature was controlled within a standard deviation of ±0.2°C. MICs were defined as the lowest drug concentration that prevented visible growth. For each day's experiments, the control organism, C. albicans ATCC 65436, was tested in SAAMF and the other media under study at 37°C with a starting inoculum of 10^6 cells per ml against each drug by the macrodilution broth method. For all studies, these standardized conditions for each drug produced results within twofold of each other.

Microdilution broth tests were performed as described previously (8, 9) under the same conditions as those used for macrodilution broth tests. Briefly, the drugs were diluted in media to two times the final concentrations, as listed above. A Biomek 1000 (Beckman Instruments, Inc., Palo Alto, Calif.) was used to serially dilute 150-μl volumes in optically standardized, flat-bottom, 96-well plates (Costar, Van Nuys, Calif.). Yeast inocula were prepared in the same manner as for the macrodilution broth method, except that the turbidimetrically adjusted suspensions were diluted 1:50 and 1:5,000. These dilutions produced final starting inocula for the various isolates ranging from 1.7 × 10^4 to 5.7 × 10^4 CFU/ml (large inocula) and from 1.1 × 10^4 to 10.1 × 10^4 CFU/ml (small inocula), as judged by quantitative subcultures of final suspensions. One hundred fifty microliters suspension was added to each well (final volume, 0.3 ml). After incubation, wells were mixed by automated pipetting (8) and MICs were read as the lowest drug concentration that prevented visible growth.

**Datum analysis.** The difference in the results for each isolate tested under each set of conditions was determined by subtracting the microdilution broth result from the macrodilution broth result. For example, the difference between a macrodilution broth MIC of 8.0 μg/ml and a microdilution broth MIC of 1.0 μg/ml would be eightfold. MICs could be determined for all combinations of test conditions for all yeast isolates except T. glabrata, for which the MIC of SCH 39304 and fluconazole for all test conditions was >512 μg/ml and the MIC of ketoconazole for the larger inoculum size was >64 μg/ml. These combinations, therefore, were excluded from the reported analyses. The values were used to calculate the mean fold difference ± the standard error of the mean. The significance of differences between means was assessed by the Mann-Whitney U test with a commercial microcomputer statistics application (Systat; Systat Inc., Evanston, Ill.), with P < 0.05 taken as significant.

**RESULTS**

**Effects of temperature, inoculum size, and medium composition on test agreement for triazoles.** When various conditions were manipulated for testing the activity of SCH 39304 and fluconazole against five yeast isolates, changes in agreement between macro- and microdilution broth results were noted (Table 1). For the two triazoles combined, agreement between the different methods for each isolate was closer at 35°C than at 37°C. The mean fold difference ± the standard error of the mean at 35°C was only 0.25 ± 0.28, while at 37°C it was 3.10 ± 0.60 (P < 0.001). The individual drugs showed similar patterns, and the differences for SCH 39304 were significant (P < 0.001).

The inoculum size also influenced agreement between macro- and microdilution broth test results. When an inoculum of 10^4 yeast cells per ml was used, the overall difference was 2.38 ± 0.61; when an inoculum of 10^5 yeast cells per ml was used, the overall difference was 0.98 ± 0.32 and was consistent for both SCH 39304 and fluconazole. When results were further stratified by temperature, differences at 37°C appeared to be significant for SCH 39304 (P < 0.02).

When temperature and inoculum size were considered in tandem, the lower temperature and the smaller inoculum size afforded the closest agreement between macro- and microdilution test results, whereas the higher temperature and the larger inoculum size produced the most discrepant results (0.25 ± 0.31 and 4.50 ± 1.04-fold differences, respectively; P < 0.001). Agreement between methods did not appear to be systematically influenced by the choice of synthetic media.

**Effects of temperature and inoculum on test agreement for other antifungal drugs.** Because of the findings with the
triazoles, we used the same panel of yeast isolates to investigate the influence of temperature and inoculum size on the agreement between macro- and microdilution MICs with commercially available antifungal agents.

Like the triazoles, amphotericin B and flucytosine also showed significantly closer agreement between methods at the lower temperature (Table 2). The mean difference for amphotericin B was more than fivefold lower at 35°C than at 37°C ($P < 0.004$). It is also of interest that the magnitude of the difference between macro- and microdilution broth MICs for amphotericin B was comparatively large, so that at 37°C microdilution MICs averaged greater than ninefold lower than macrodilution MICs. Flucytosine had a narrow range of variation overall. However, at 35°C the mean difference between methods was 0.17 ± 0.39, a value which was significantly different from −1.50 ± 0.61 with incubation at 37°C ($P < 0.05$). In contrast to the other antifungal agents, ketoconazole produced less agreement at 35°C than at 37°C (mean 4.00 ± 1.45- and 0.73 ± 1.36-fold differences, respectively), although this effect did not reach a critical level of significance.

For all three drugs, inoculum size appeared to exert little influence on agreement.

### DISCUSSION

In this study, the variables of incubation temperature and yeast inoculum size both were found to affect the agreement between macro- and microdilution endpoints of one or another antifungal agent. With four of the five drugs tested, incubation at 35°C produced better agreement than did incubation at 37°C. In contrast, agreement with ketoconazole was better at the higher temperature, although the effect was not consistent among isolates and the significance will need to be confirmed by further study. Inoculum size also was found to affect both flucytosine and SCH 39034 but not amphotericin B, flucytosine, or ketoconazole to a demonstrably significant degree. Moreover, with the triazoles, the effect of inoculum size appeared to be additive with that of temperature, so that a small inoculum and a low temperature produced the best agreement and a large inoculum and a high temperature produced the worst.

There is little published information regarding systematic differences between macro- and microdilution broth test results for yeasts under different test conditions. In one report with antibacterial agents, microdilution MICs were found to be slightly lower than macrodilution MICs with coliforms but not with gram-positive aerobic cocci (1). The authors of that report suggested that the differences might have been due to a decreased ability to detect turbidity in the microdilution wells or differences in the absolute number of microorganisms within wells of a microdilution tray as compared with macrodilution tubes. Presumably these same differences could account for our findings as well.

Regardless of the reasons for the temperature effects, these observations are pertinent to efforts to reconcile endpoint results produced by different methods of testing antifungal agents. Since our findings indicate that agreement is better for most antifungal agents at 35°C, this incubation temperature would seem preferable for standardized methods. This is fortuitous, since this temperature is conventionally used in many clinical laboratories. Moreover, in recently completed collaborative studies, incubation at 35°C was found to support the growth of several yeast isolates better than incubation at 30°C (M. A. Pfaller, M. G. Rinaldi, J. N. Galgiani, M. S. Bartlett, B. A. Body, A. Espinel-Ingroff, R. A. Fromling, G. S. Hall, C. E. Hughes, F. C. Odds, and A. M. Sugar, Antimicrob. Agents Chemother., in press). Finally, although in our research laboratory setting relatively strict incubation temperature control was possible, such control may be harder to achieve in a clinical laboratory setting and conceivably in practice could contribute to discrepancies between macro- and microdilution results.

Similarly, our findings suggest that a yeast inoculum size smaller than 10⁵ cells per ml may also improve the agreement of results with some triazoles. However, further studies with larger numbers of yeast isolates will be needed to assess whether selecting these conditions will produce adequate agreement among laboratories to merit their endorsement in standardized procedures.

### ACKNOWLEDGMENT

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