Multicenter Evaluation of a Broth Macrodilution Antifungal Susceptibility Test for Yeasts

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Received 15 July 1992/Accepted 2 October 1992

Thirteen laboratories collaborated to optimize interlaboratory agreement of results of a broth macrodilution procedure for testing three classes of antifungal drugs against pathogenic yeasts. The activities of amphotericin B, flucytosine, and ketoconazole were tested against 100 coded isolates of Candida albicans, Candida tropicalis, Candida parapsilosis, Candida lusitaniae, Torulopsis (Candida) glabrata, and Cryptococcus neoformans. Two starting yeast inoculum sizes (5 × 10^4 and 2.5 × 10^5 cells per ml) were compared, and readings were taken after 24 and 48 h of incubation. All other test conditions were standardized. The resultant turbidities in all tubes were estimated visually on a scale from 0 to 4+ turbidity, and MIC-0, MIC-1, and MIC-2 were defined as the lowest drug concentrations that reduced growth to 0, 1+, or 2+ turbidity, respectively. For flucytosine, agreement among laboratories varied between 57 and 87% for different inocula, times of incubation, and end point criteria. Agreement was maximized (85%) when the lower inoculum was incubated for 2 days and the MICs were defined as 1+ turbidity or less. For amphotericin B, variations in test conditions produced much smaller differences in interlaboratory agreement. For ketoconazole, interlaboratory agreement was poorer by all end point criteria. However, MIC-2 endpoints distinguished T. glabrata as resistant compared with the other species. Overall, the studies indicated that readings from the lower inoculum obtained on the second day of reading result in the greatest interlaboratory agreement. In combination with data from previous multicenter studies (National Committee for Clinical Laboratory Standards, Antifungal Susceptibility Testing: Committee Report, Vol. 5, No. 17, 1988; M. A. Pfaller, L. Burmeister, M. S. Bartlett, and M. G. Rinaldi, J. Clin. Microbiol. 26:1437-1441, 1988; M. A. Pfaller, M. G. Rinaldi, J. N. Galgiani, M. S. Bartlett, B. A. Body, A. Espinel-Ingroff, R. A. Fromting, G. S. Hall, C. E. Hughes, F. C. Odds, and A. M. Sugar, J. Clin. Microbiol. 34:1648-1654, 1996), these findings will be used by the National Committee for Clinical Laboratory Standards to develop a standardized method for in vitro antifungal susceptibility testing for yeasts.

Fungal infections have emerged as major causes of morbidity and mortality in compromised patient populations. During the last 15 years, the incidence of fungal infections has continued to increase in part because of improved diagnosis and the increasing number of patients with AIDS, organ transplants, malignancies, and other immunocompromising factors (4, 40). The frequency of fungal infections has greatly increased interest in testing new fungal isolates from patients by clinical laboratories, and efforts to develop standardized methodologies for such tests have been reported (18, 27, 33, 39). In addition, an increasing number of reports suggest that clinically significant resistance exists in some strains (13-15, 19, 22, 37, 38, 43). Standardized methods of testing isolates would permit the development of a more reliable data base from which to study this clinically relevant issue.

Since standardization is vital to meaningful communication between clinical laboratories and physicians and to the application of in vitro data to patient responses to therapy, the National Committee for Clinical Laboratory Standards formed a subcommittee on antifungal susceptibility testing in 1982. Four years later, the first subcommittee report was published in which the results of a questionnaire and small collaborative study were presented (5, 24). Among the findings were that most centers used a broth dilution meth-
odology, only a few isolates were tested annually, Candida albicans or other species of yeasts were the most commonly tested species of fungi, and MIC data among the laboratories that participated in the collaborative study were unaccept-
ably low. Two subsequent multicenter studies (30, 32) have focused on inoculum preparation, inoculum size, selection of synthetic media, temperature and duration of incubation, and end point definition. These studies established criteria for inoculum preparation by using a spectrophotometric method (30), selection of RPMI 1640 medium (32), and an incubation temperature of 35°C (32) for antifungal susceptibility testing of yeasts.

The present collaborative study was performed to evaluate the utility of RPMI 1640 medium buffered with morpholinepropanesulfonic acid (MOPS) and an incubation temperature of 35°C in a broth macrodilution susceptibility test method with a panel of 100 pathogenic yeast isolates and three classes of antifungal agents. The effects of inoculum size and incubation times of 24 and 48 h on the ability to determine the susceptibilities of isolates of Candida species, Torulopsis (Candida) glabrata, and Cryptococcus neoformans also were assessed. The data obtained in the present study, when combined with the information gained in the previous studies of the National Committee for Clinical Laboratory Standards (5, 24, 30, 32), have supported the development of a proposed standardized method for in vitro antifungal susceptibility testing of yeasts.

MATERIALS AND METHODS

Antifungal agents. The following three antifungal agents were used: amphotericin B (Bristol Myers-Squibb), fluconazole (Systat; Systat Inc., Evanston, Ill.) containing dilutions of one of the antifungal agents (0.1 ml at a 10-fold final concentration) by using sterile, individual 1-m1 pipettes. Each yeast isolate was tested at two inocula, and drug-free and yeast-free control tubes were included. The contents of the tubes were mixed, and racks of loosely capped tubes were incubated without agitation in air at 35°C.

Tubes were examined on the first and second days on which significant growth was evident in the drug-free control tube. For species other than C. neoformans, this was after 24 and 48 h of incubation. For nearly all isolates of C. neoformans, this was after 48 and 72 h of incubation. At the times of this examination, each tube was flicked gently and the turbidity was estimated by using the following scale: 0, optically clear; 1+, slightly hazy; 2+, prominent reduction in turbidity compared with that of the drug-free control; 3+, slight reduction in turbidity compared with that of the drug-free control; 4+, no reduction in turbidity compared with that of the drug-free control. Investigators recorded the estimated turbidity for all tubes on standardized data sheets.

Study design and analysis of results. Each of the 13 participating laboratories received a protocol, antifungal drugs, media, coded yeast isolates, and standardized data sheets. When tests were completed, the data sheets were forwarded to one center where the results were entered into a microcomputer data base (Rhase; Micromir Inc., Redmond, Wash.), and the data were subsequently transferred to a statistical program (Systat; Systat Inc., Evanston, Ill.) for analysis. Listings of the entered data were returned to the reporting laboratory, where the accuracies of the entries were checked and errors were corrected.

The visual estimates of turbidity were used to calculate different end point results. MIC-0, MIC-1, and MIC-2 were
TABLE 1. Percent agreement of different yeast strains for amphotericin B results with low and high starting inoculum sizes and first and second day readings

<table>
<thead>
<tr>
<th>Species (no. of tests)</th>
<th>MIC-0</th>
<th></th>
<th>MIC-1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% within twofold of mode for*</td>
<td>% within twofold of mode for*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>C. albicans (1,761)</td>
<td>85 (0.98)</td>
<td>87 (0.98)</td>
<td>90 (0.99)</td>
<td>86 (0.99)</td>
</tr>
<tr>
<td>C. lusitaniae (520)</td>
<td>83 (0.94)</td>
<td>84 (0.96)</td>
<td>85 (0.99)</td>
<td>83 (0.99)</td>
</tr>
<tr>
<td>C. neoformans (597)</td>
<td>69 (0.92)</td>
<td>78 (0.95)</td>
<td>81 (1.0)</td>
<td>79 (1.0)</td>
</tr>
<tr>
<td>C. parapsilosis (768)</td>
<td>82 (0.95)</td>
<td>85 (0.95)</td>
<td>90 (0.99)</td>
<td>87 (0.98)</td>
</tr>
<tr>
<td>C. tropicalis (724)</td>
<td>85 (0.99)</td>
<td>89 (0.99)</td>
<td>92 (0.99)</td>
<td>91 (1.0)</td>
</tr>
<tr>
<td>T. glabrata (480)</td>
<td>90 (0.99)</td>
<td>93 (0.99)</td>
<td>95 (1.0)</td>
<td>87 (1.0)</td>
</tr>
</tbody>
</table>

* Values in parentheses are the proportion of results that were within the drug concentration range and included in the analysis. Low and high indicate inoculum sizes.
species and test condition combinations, agreement was as low as 19%. Overall, MIC-2 end points were better than either MIC-0 or MIC-1 end points, and day 2 low-inoculum results displayed significantly better agreement than those obtained with other test conditions (Table 4).

**Distribution of susceptibility results by different end points.**

Since optimal conditions for broth macrodilution testing appeared to include low inoculum size and readings on day 2, modal results for those conditions for each strain were used to construct susceptibility distributions for each drug and species tested. For amphotericin B, the MIC-0 end points, which were equivalent to MIC-1 results, are shown in Fig. 1. There was remarkable homogeneity of results for all species, and the MIC-0 for all strains ranged from 0.25 to 1.0 μg/ml. A more resistant cluster was not detected in this group of isolates.

For 5-FC, MIC-1 results, which showed better agreement than MIC-0 results, are shown in Fig. 2. The predominant distribution of results ranged from ≤0.125 to 8 μg/ml. However, in four of the species tested, for one or more isolates MIC-1 results were >64 μg/ml. This result was from 16- to 1,024-fold greater than those for other isolates of the same species, thus suggesting a more resistant grouping above that for the norm.

For ketoconazole, all three endpoints are shown in Fig. 3 and 4, and the modal distributions demonstrated sharply different patterns. MIC-0 results were uniformly higher, most notably for *C. albicans*, *C. tropicalis*, and *T. glabrata*. With MIC-1 and MIC-2 results, end points shifted dramatically lower for *C. albicans* and *C. tropicalis*, whereas *T. glabrata* showed much less of a shift. As a result, by using the MIC-2 end point, for 70% of the *T. glabrata* isolates the MIC-2 was ≥2.0 μg/ml, whereas for all strains of other species, the MIC-2 was ≤1.0 μg/ml.

**DISCUSSION**

In the present study, results produced with the lower inoculum and interpreted on the second day of readings proved to be significantly more reproducible among laboratories than those found with either the higher inoculum or first day readings. Under optimal conditions, 90% of amphotericin B results for each strain from different laboratories agreed within a fourfold range regardless of the end point

<table>
<thead>
<tr>
<th>Species (no. of tests)</th>
<th>% within twofold of mode for*:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1 Low</td>
</tr>
<tr>
<td><em>C. albicans</em> (1,762)</td>
<td>39 (0.23)</td>
</tr>
<tr>
<td><em>C. lusitaniae</em> (519)</td>
<td>55 (0.72)</td>
</tr>
<tr>
<td><em>C. neoformans</em> (597)</td>
<td>49 (0.80)</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (768)</td>
<td>69 (0.80)</td>
</tr>
<tr>
<td><em>C. tropicalis</em> (724)</td>
<td>35 (0.68)</td>
</tr>
<tr>
<td><em>T. glabrata</em> (480)</td>
<td>50 (0.85)</td>
</tr>
<tr>
<td><em>A. niger</em> (1,708)</td>
<td>83 (0.16)</td>
</tr>
</tbody>
</table>

* Values in parentheses are the proportion of results that were within the drug concentration range and included in the analysis. Low and high indicate inoculum sizes.

**TABLE 4.** Overall percent agreement of MIC-0, MIC-1, and MIC-2 end points for low and high starting inoculum sizes read on days 1 and 2*

<table>
<thead>
<tr>
<th>Drug</th>
<th>End point</th>
<th>% Agreement on day 1*</th>
<th>Significance</th>
<th>% Agreement on day 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>MIC-0 (4,253)</td>
<td>85</td>
<td>87</td>
<td>←0.03→</td>
</tr>
<tr>
<td></td>
<td>MIC-1 (4,253)</td>
<td>81</td>
<td>86</td>
<td>←0.002→</td>
</tr>
<tr>
<td>5-FC</td>
<td>MIC-0 (3,257)</td>
<td>66</td>
<td>68</td>
<td>←0.001→</td>
</tr>
<tr>
<td></td>
<td>MIC-1 (2,574)</td>
<td>57</td>
<td>83</td>
<td>NS*</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>MIC-0 (2,573)</td>
<td>43</td>
<td>48</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>MIC-1 (3,800)</td>
<td>46</td>
<td>42</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>MIC-2 (2,656)</td>
<td>52</td>
<td>69</td>
<td>←0.02→</td>
</tr>
</tbody>
</table>

* By a chi-square test of significance of differences among the four sets of conditions for each drug and end point, *P* values were all <0.004.

* Values in parentheses are the number of results (results for *C. neoformans* and offscale results were deleted).

* Significance of differences between day 1 high-inoculum and day 2 low-inoculum results are provided, but a Bonferroni correction was not applied.

* NS, not significant.
criterion used. However, with 5-FC and ketoconazole, agreement was improved with MIC-1 and MIC-2, respectively.

The reason that the less stringent end points improved agreement for these two drugs is likely related to the trailing nature of the drug effect documented in past studies; this also would be expected to be evident with other azole congeners such as miconazole and fluconazole (11, 16, 26, 28, 36). The titration curve for the effect of amphotericin B is much sharper than exists with the other drugs studied. In addition, the onset of the effect of 5-FC and azoles is delayed, allowing growth to proceed for some period before inhibition is initiated (17, 23). In antibacterial susceptibility testing, an analogous problem has been encountered for sulfa drugs tested by an agar diffusion test; in that circumstance an end point criterion of 80% inhibition has been adopted (2, 3).

Although not specifically examined in the present study, recent parallel studies reported elsewhere (10) have determined that the estimates of turbidity described here as 1+ and 2+ can be approximated by 1:10 and 1:5 dilutions of the drug-free control, respectively. This approach provides a convenient and direct method of establishing a turbidity end point for a specific isolate that precisely reflects 90 and 80% inhibition, respectively, and further reduces the likelihood of interobserver error. The need for disregarding slight amounts of turbidity for some antifungal agents is related to the person making the reading as well as to other factors, including incomplete drug solubility.

In addition to interlaboratory agreement, an important consideration is the effect that different test conditions have on identifying potentially resistant strains. The isolates of C. neoformans were selected with this in mind for 5-FC; the putatively resistant isolates could be distinguished as resistant under the optimized procedure. However, the susceptibility of C. neoformans to other drugs and the susceptibilities of the other species to any of the drugs tested were not used as criteria to select isolates. On the basis of the assumption that isolates of most Candida species are susceptible to amphotericin B, 5-FC, and azoles, it would be expected that most isolates would constitute a susceptible distribution. On the basis of this assumption, we propose that the upper limits of susceptibility test results by the
optimized method be 1.0, 8.0, and 0.25 μg/ml for amphotericin B, 5-FC, and ketoconazole, respectively. In the absence of clinical data, determination of breakpoint values could be proposed by using both pharmacokinetic and population distribution data. This approach will require a study with a greater number of strains. Ultimately, however, clinical correlation will be essential for validating any breakpoint for this method.

By using the MIC-2 criterion for ketoconazole, the distribution of modal susceptibilities for T. glabrata indicated that it was notably more resistant than other species. This is in keeping with results of previous in vitro studies and several clinical observations with ketoconazole and other azoles (20, 21, 41, 42). In contrast, the MIC-0 criterion did not distinguish T. glabrata from other species. Not tested in this study but of additional interest would be the MICs for isolates of Candida krusei, which has also been associated withazole resistance (1, 6, 8, 12, 29, 35, 44). In a future study, it will be important to determine whether the resistance of this species can be identified by the MIC-2 criterion as well.

The amphotericin B susceptibility of C. lusitaniae noted in our results also is of special interest, since a few isolates of this species from some institutions have been reported to be resistant to amphotericin B (7). All of the strains of this species tested in the present study appeared to have susceptibilities identical to those of the other species tested, and this raises the possibility that in attaining optimal interlaboratory agreement, the procedure has become insensitive to potentially important amphotericin B resistance. This important issue needs to be addressed in future studies.

This and previous collaborative studies (24, 30, 32) done in coordination with the National Committee for Clinical Laboratory Standards have resulted in demonstrable progress in standardizing antifungal susceptibility testing of yeasts. Although excellent multicenter studies have been done by others (18, 27, 33, 39), none of them have been developed into a widely used standardized procedure for a range of chemical classes of antifungal drugs. The data obtained in the present study, in combination with data from previous multicenter studies (24, 30, 32), have been used by the National Committee for Clinical Laboratory Standards in the development of a standardized method for in vitro antifungal susceptibility testing for yeasts (25a). It is hoped that the procedure will provide a tool for determining the clinical utility of testing for the clinician. In addition to drug susceptibility, other factors, such as anatomy, immunologic competence, or drug distribution and metabolism, are critical for estimating clinical responses in patients. Future studies are needed to define the significance of in vitro test results in relation to these other factors and how in vitro tests should be used to guide therapy.

ACKNOWLEDGMENTS

We acknowledge the contributions of G. Abruzzo, S. Dufresne, A. Fothergill, C. Gill, D. McCough, C. Trainor, and the many research assistants, fellows, and technologists who supported this project at the 13 sites. The statistical consultation of G. A. Cloud, made available by the NIAID Mycoses Study Group (N01-AI-15082), is gratefully appreciated.

This project was supported in part by the U.S. Department of Veterans Affairs and by grants from Bristol Myers-Squibb, Merck & Co., Inc., Pfizer Pharmaceuticals, and SmithKline Beecham. Bristol Myers-Squibb, Hoffmann-La Roche Laboratories, and Janssen Pharmaceuticals provided antifungal reagents for the study.

REFERENCES


