Antifungal Susceptibility Testing: Technical Advances and Potential Clinical Applications

M. A. Pfaffer, J. H. Rex, and M. G. Rinaldi

The clinical application of in vitro antifungal susceptibility testing has been limited by a lack of reproducibility and uncertain clinical relevance. As a result of several collaborative studies, the National Committee for Clinical Laboratory Standards (NCCLS) has proposed a standardized antifungal susceptibility test method, NCCLS M27-T. More convenient, user-friendly methods (microdilution broth and stable gradient technology) have been evaluated, and the potential for a similar process with a disk diffusion method is apparent. Adaptation of the standard method for susceptibility testing of filamentous fungi appears promising. The existence of a standardized method facilitates meaningful analysis of studies addressing the issue of clinical relevance of antifungal susceptibility testing. Correlation of MICs with clinical response to therapy is beginning to emerge, most notably in relation to fluconazole and itraconazole therapy for oropharyngeal candidiasis associated with infection with the human immunodeficiency virus. This accumulated experience with antifungal susceptibility testing allows us to provide several specific recommendations for antifungal susceptibility testing in the clinical laboratory. Application of this developing technology to new antifungal agents and other disease states will enhance our ability to effectively deal with the emerging problem of fungal infection.

The primary objective of virtually all in vitro antimicrobial susceptibility testing is to aid in the prediction of the effect of the antimicrobial agent of interest on the outcome of infection caused by a specific pathogen. This is true whether or not the in vitro susceptibility tests are being done for patient care, for drug development or drug discovery, or in epidemiological studies. Regardless of the purpose of antimicrobial susceptibility testing, results obtained in a simple, well-defined, and highly artificial in vitro test system have intrinsic limitations in predicting the outcome of the complex biological processes that clinical infections represent. Given these limitations, it is understandable that only modest correlation exists between in vitro test results and clinical outcome despite decades of experience with standardized testing methods [1–3].

Efforts in the United States to develop standardized and clinically useful methods for in vitro susceptibility testing of antifungal agents began in 1982 with the establishment of the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee for Antifungal Susceptibility Testing. At that time, it was apparent that antifungal susceptibility testing was decades behind that of antibacterial testing. Among the reasons for this slow development were the relative infrequency of fungal infections before the 1980s as well as the limited number of available therapeutic agents. Furthermore, the potential for the emergence of resistance to antifungal agents was not well-recognized.

Clearly, this has all changed. Over the past decade, the increased importance of fungal infections in both the hospital and outpatient setting has become well-recognized [4–6]. Likewise, the pharmaceutical industry has responded to the increased need for therapeutic agents with the development of several new antifungal agents for use in the treatment of both systemic and superficial fungal infections [7].

Finally, it is now readily apparent that resistance to antifungal agents is an issue of both clinical and epidemiological importance [8–14]. These factors have created a need for standardized, reproducible, and clinically relevant in vitro antifungal susceptibility testing methods to help guide therapeutic decision-making, to aid in drug development studies, and as a means of tracking the development of antifungal resistance in epidemiological studies [9, 12, 15].

In response to this need, a number of investigators have worked both independently and in collaboration with the NCCLS subcommittee to investigate the key in vitro testing variables of inoculum preparation and size, medium composition, temperature and duration of incubation, and MIC endpoint determination in an effort to develop a standardized approach to antifungal susceptibility testing [16–26]. These and other
collaborative studies over the past decade have allowed the NCCLS Subcommittee on Antifungal Testing to achieve consensus and to propose a standardized broth dilution method for in vitro testing of antifungal agents [12, 27]. The NCCLS reference method has been published as document M27-T [28] and provides a standardized guideline for the performance of both macrodilution and microdilution broth susceptibility testing of clinical yeast isolates.

The NCCLS M27 methodology has been reviewed in detail in previous publications [7, 12, 27], and the key features of the method are summarized in table 2. Adherence to NCCLS M27-T provides >90% intralaboratory and interlaboratory reproducibility [16, 18, 29], and the use of recommended quality-control isolates will further ensure reliable test performance [28, 30, 31]. Thus, considerable progress has been made since 1982 in the development of standardized methods for the performance of antifungal susceptibility testing (table 2).

Given the availability of a standardized reference method, it is now feasible to develop alternative testing methods that are better adapted for use in the clinical laboratory. The availability of standardized methods facilitates clinical investigations designed to determine the clinical utility of in vitro antifungal susceptibility testing [8]. In the remainder of this update we provide an overview of technical issues that are currently being addressed and discuss several issues relevant to the clinical application and interpretation of antifungal susceptibility testing.

### Technical Issues in Antifungal Susceptibility Testing

**Advances**

**Alternatives to macrodilution testing** As indicated in table 2, the first method developed by the NCCLS subcommittee was a macrodilution broth method for *Candida* and *Cryptococcus* species. Although useful as a reference method, macrodilution testing is too cumbersome for the clinical microbiology laboratory. Using the macrodilution method of M27 as a touchstone, several investigators have demonstrated the comparability of more user-friendly methods such as microdilution broth [16, 17, 25, 32-38] and stable gradient agar diffusion (Etest; AB BIODISK, Solna, Sweden) [38-43].

These methods employ technology that is used routinely in clinical microbiology laboratories for antibacterial testing and may be read visually, spectrophotometrically, or colorimetrically as a means of improving the accuracy and reproducibility of the MIC endpoint (table 3). Both microdilution and Etest are much more practical and easier to perform than the macrodilution method, and the results generally agree quite well with those of the more cumbersome reference method (table 3). The microdilution broth method has been included in the NCCLS M27-T document [28] as an acceptable alternative to the macrodilution method for use in the clinical laboratory.

Although used extensively in the clinical laboratory for antibacterial testing, disk diffusion testing has had limited application in antifungal susceptibility testing. Several studies have shown that disk diffusion testing of fluconazole, which is used extensively in Europe, may be useful [22, 44-46]. Both Barry and Brown [44] and Troillet et al. [45] have shown that findings

### Table 1. Key features of NCCLS M27 methodology for antifungal susceptibility testing.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>Macrodilution (final volume, 1 mL)</td>
</tr>
<tr>
<td></td>
<td>Microdilution (final volume, 200 µL)</td>
</tr>
<tr>
<td>Inoculum preparation</td>
<td>Spectrophotometric adjustment with use of 0.5 McFarland BaSO₄ turbidity standard</td>
</tr>
<tr>
<td>Inoculum concentration</td>
<td>0.5–2.5 × 10⁵ cells/mL</td>
</tr>
<tr>
<td>Test medium</td>
<td>RPMI 1640</td>
</tr>
<tr>
<td>Buffer</td>
<td>Morpholinelopaneptosulfonic acid (MOPS), 0.165 M</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
<tr>
<td>Incubation</td>
<td>35°C</td>
</tr>
<tr>
<td>Time</td>
<td>48 h (72 h for <em>Cryptococcus neoformans</em>)</td>
</tr>
<tr>
<td>MIC endpoint determination</td>
<td>Amphotericin B: no visible turbidity</td>
</tr>
<tr>
<td></td>
<td>Azoles and fluconosine: &gt;80% inhibition</td>
</tr>
</tbody>
</table>

NOTE. Method is published in NCCLS document M27-T [28]. Table is modified from [14].

### Table 2. Progress since 1982 in the development of antifungal susceptibility testing methods.

1. Development of macrodilution reference method for *Candida* and *Cryptococcus* species
2. Establishment of quality-control strains and MIC reference ranges for amphotericin B, fluconazole, itraconazole, and ketoconazole
3. Development of alternative methods
   a. Microdilution broth method
   b. Spectrophotometric endpoint determination
   c. Colorimetric endpoint determination
   d. Stable gradient agar MIC test (Etest)
   e. Disk diffusion test (fluconazole)
4. Standardized method for testing filamentous fungi

### Table 3. Alternative methods for antifungal susceptibility testing.

<table>
<thead>
<tr>
<th>Testing method</th>
<th>Method of endpoint determination</th>
<th>Agreement (%) of results with those of macrodilution reference method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microdilution</td>
<td>Visual</td>
<td>&gt;90</td>
</tr>
<tr>
<td></td>
<td>Spectrophotometric</td>
<td>89–100</td>
</tr>
<tr>
<td></td>
<td>Colorimetric</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Etest</td>
<td>Visual</td>
<td>76–100</td>
</tr>
<tr>
<td>Disk diffusion</td>
<td>(fluconazole only)</td>
<td>Visual</td>
</tr>
</tbody>
</table>
of the fluconazole disk test correlate quite well with broth dilution MICs; however, Barry and Brown [44] found that the disk test did not adequately separate fully resistant strains from those with intermediate or dose-dependent susceptibility. This approach will require additional development but should prove to be quite useful as a very simple means of performing fluconazole susceptibility testing.

Susceptibility testing of filamentous fungi. Efforts are now under way to address the issue of in vitro susceptibility testing of filamentous fungi (molds). Given the extensive phenotypic variation observed among the molds, it is expected that this will be a major challenge. Despite these reservations, the NCCLS subcommittee has proceeded in the hope that standardization of the various antifungal susceptibility testing steps for yeasts has opened the possibility of developing similar standards for the molds.

As such, the NCCLS subcommittee has adapted the M27 methodology to accommodate the use of a conidial suspension as the starting inoculum and has proceeded with multicenter studies to assess the issues of intralaboratory and interlaboratory reproducibility, the use of macrodilution and microdilution broth methods, and in vivo correlation. The initial collaborative six-center study evaluated the use of macrodilution and microdilution broth methods to determine the in vitro susceptibility of 25 isolates of filamentous fungi (Aspergillus fumigatus, Aspergillus flavus, Pseudallescheria boydii, Rhizopus arrhizus, and Sporothrix schenckii) to amphotericin B, fluconazole, itraconazole, miconazole, and ketoconazole [47].

The results of this study were very encouraging and demonstrated excellent intralaboratory and interlaboratory agreement (90%–100%) for both macrodilution and microdilution methods in testing of amphotericin B, fluconazole, miconazole, and ketoconazole. A lower level of agreement (70%–90%) was observed in the testing of itraconazole.

On the strength of these results, a subsequent 11-laboratory study was conducted in which a microdilution method was employed to test amphotericin B and itraconazole against 30 isolates, including A. flavus, A. fumigatus, Fusarium oxysporum, Fusarium solani, P. boydii, and R. arrhizus [48]. In this study the MIC endpoints were determined visually, with and without a colorimetric indicator. The results of this study were also quite encouraging and demonstrated an interlaboratory agreement of 94%–96% for amphotericin B and 89%–95% for itraconazole.

Thus, significant progress has been made in adapting the M27 methodology to accommodate the testing of filamentous fungi. The excellent intralaboratory and interlaboratory reproducibility observed with the microdilution method is encouraging and will facilitate future studies evaluating the in vivo correlation of this in vitro test method.

Ongoing Problems

In vitro testing of amphotericin B. It is now well-recognized that the use of NCCLS M27-T methodology to test amphotericin B against Candida species results in a very narrow range of MIC values (0.12–2.0 μg/mL) [12, 43, 49]. Given these results, there is concern that the M27 methodology may mask clinically relevant variations in amphotericin B susceptibility [12, 49].

Rex et al. [49] used a panel of well-characterized amphotericin B–resistant strains of Candida species to demonstrate that this was in fact true. Although the MICs of amphotericin B (2.0 μg/mL) were generally higher for resistant strains than for susceptible strains when tested by M27 methodology, the difference was usually no more than one or two log dilutions and thus within the limits of variation of the test method.

The difference in amphotericin B susceptibility between resistant and susceptible strains was more pronounced when antibiotic medium 3 broth was used in place of the standard RPMI 1640 broth [49] or when the isolates were tested by Etest with use of RPMI 1640 agar [43]. Thus, it appears that the M27 test method must be modified slightly in order to optimize the detection of amphotericin B resistance. While this modification is noted in the NCCLS M27-T document [28], the reproducibility and proper interpretation of results obtained with this medium is still under study.

Cryptococcus neoformans. Throughout the development of the M27 methodology, it was clear that this approach was suboptimal for the testing of C. neoformans [12]. Although reliable and reproducible MIC results can be generated for most strains of C. neoformans, growth in the RPMI 1640 broth is slow, requiring incubation times of 72 hours, and some strains simply may not grow in this medium.

A modification of the NCCLS M27 method was proposed by Ghannoum et al. [50] and has proved to be an acceptable alternative to the reference method [51, 52]. In this method, yeast nitrogen base medium buffered to a pH of 7.0, an inoculum of 10^4 cells/mL, and incubation at 35°C for 48 hours are used in a microdilution format [50]. The MIC endpoint is read spectrophotometrically and for fluconazole is defined as 50% inhibition at 420 nm.

The advantages of this approach include better growth of clinical isolates in yeast nitrogen base medium, good reproducibility, and the ability to provide a range of fluconazole MICs that generally reflect the clinical response to this agent [52]. A recent multicenter evaluation of this method demonstrated an excellent level of interlaboratory agreement (96%) and an overall agreement of 90% with the M27-T microdilution method [51].

Given these results, it appears that the method of Ghannoum et al. [50] is superior to the NCCLS reference method for susceptibility testing of C. neoformans against fluconazole. However, additional studies will be necessary to standardize this method and to allow its use in testing of C. neoformans against other antifungal agents.

Clinical Application and Interpretation of Antifungal Susceptibility Tests

Ideally, the results of in vitro antimicrobial susceptibility tests should provide a reliable prediction of in vivo response
to therapy in human infections. However, as noted previously, the limitations of these highly artificial testing methods are such that only modest correlation exists between in vitro susceptibility testing results and clinical outcome. Although in vitro resistance frequently (but not always) predicts clinical failure, in vitro susceptibility does not always predict successful therapy [14, 53].

This point is well-illustrated when one considers the data supporting the MIC interpretive breakpoints for the antibacterial agent cefotaxime (table 4). Although there is a clear relationship between clinical cure and MIC, not all patients infected with susceptible organisms were cured and not all those infected with resistant organisms failed to respond to therapy with cefotaxime. It is useful to keep these caveats in mind as we consider the clinical relevance of in vitro antifungal susceptibility testing.

The availability of microdilution adaptations of the NCCLS M27 reference method has facilitated some large-scale surveys of the in vitro susceptibility of Candida isolates to commonly used antifungal agents. Such surveys have been useful in generating broad MIC distribution profiles for clinical isolates (table 5).

As these and other studies have demonstrated species-specific differences among Candida isolates in susceptibility to the various antifungal agents (table 6), the generation of antifungal susceptibility profiles coupled with the identification of fungal pathogens to the species level may provide useful clinical and epidemiological markers for the presence of potentially resistant strains of Candida species [54–64]. Such an approach could be part of broader periodic surveillance efforts to detect antimicrobial resistance (both bacterial and fungal) within a given institution.

### Candida Species

The greatest effort in establishing the in vivo correlation of antifungal susceptibility testing has been directed toward Candida species [8, 13, 14]. Data are now beginning to accumulate for three antifungal agents: amphotericin B, fluconazole, and itraconazole.

**Amphotericin B.** As noted previously, resistance to amphotericin B is unusual. Although 94%–99% of Candida species isolates are inhibited by a ≤1.0—1μg/mL concentration of amphotericin B (table 5), there is concern that the NCCLS M27-T method may be insensitive to amphotericin B resistance [12, 13, 43, 49, 65]. This concern was raised specifically in a recent study of candidemia in nonneutropenic patients [65]. The amphotericin B MICs, determined for these isolates by NCCLS M27 methods, were all ≤1.0 μg/mL, and thus the clinical failures were all associated with the isolates with low MICs.

This apparent lack of correlation with clinical outcome raises the question of a problem with the in vitro testing method, vs. the likelihood that factors other than intrinsic antifungal susceptibility were more important in determining the outcome. It is notable that repeated testing of these isolates with methods known to be capable of detecting amphotericin B–resistant strains (antibiotic medium 3 and Etest) also failed to identify those associated with elevated amphotericin B MICs among this population [43, 49].

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**Table 4.** Correlation between cefotaxime susceptibility test results and clinical response.

<table>
<thead>
<tr>
<th>MIC (μg/mL)</th>
<th>Interpretation</th>
<th>No. of patients</th>
<th>Percentage cured</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤8.0</td>
<td>Susceptible</td>
<td>1,440</td>
<td>93</td>
</tr>
<tr>
<td>16–32</td>
<td>Intermediate</td>
<td>99</td>
<td>78</td>
</tr>
<tr>
<td>≥64</td>
<td>Resistant</td>
<td>26</td>
<td>65</td>
</tr>
</tbody>
</table>

NOTE. Table is adapted from [2].

**Table 5.** In vitro susceptibility of clinical yeast isolates to three antifungal agents, as determined by NCCLS microdilution broth methods.

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>No. of isolates</th>
<th>MIC (μg/mL): % of isolates inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>597</td>
<td>≤1.0: 94.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥2.0: 6.0</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>597</td>
<td>≤8.0: 91.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16–32: 6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥64: 2.0</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>597</td>
<td>≤8.0: 96.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16–32: 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥64: 1.9</td>
</tr>
</tbody>
</table>

NOTE. Table is adapted from [35, 54].

**Table 6.** Typical fluconazole and itraconazole MICs for Candida species isolates.

<table>
<thead>
<tr>
<th>Candida species</th>
<th>No. of isolates tested</th>
<th>Antifungal agent</th>
<th>Range (μg/mL)</th>
<th>MIC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>albicans</td>
<td>160</td>
<td>Flu</td>
<td>0.12–256</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Itr</td>
<td>0.015–16</td>
<td>0.12</td>
</tr>
<tr>
<td>glabrata</td>
<td>75</td>
<td>Flu</td>
<td>0.25–&gt;128</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Itr</td>
<td>0.06–8</td>
<td>2</td>
</tr>
<tr>
<td>tropicalis</td>
<td>89</td>
<td>Flu</td>
<td>0.25–8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Itr</td>
<td>0.03–1</td>
<td>0.5</td>
</tr>
<tr>
<td>parapsilosis</td>
<td>60</td>
<td>Flu</td>
<td>0.12–8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Itr</td>
<td>0.03–0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>krusei</td>
<td>67</td>
<td>Flu</td>
<td>16–&gt;128</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Itr</td>
<td>0.5–2</td>
<td>1</td>
</tr>
</tbody>
</table>

NOTE. Flu = fluconazole; Itr = itraconazole. Table is adapted from multiple sources [54–56, 58–61, 64].
Thus, although the NCCLS M27 method may require modification to provide optimal detection of amphotericin B resistance, resistance per se appears to be uncommon, and clinical failure of amphotericin B therapy for candidiasis is more likely due to host factors than to intrinsic or acquired antifungal resistance.

**Fluconazole.** Fluconazole is used widely for the treatment of both superficial and deep-seated candidiasis. Although resistance to fluconazole remains uncommon, its occurrence among isolates of *Candida* species, particularly *Candida albicans*, *Candida glabrata*, and *Candida krusei*, has been well documented [8, 13]. It has become increasingly clear that the NCCLS M27 methodology detects intrinsic differences in susceptibility of *Candida* isolates to fluconazole in animal models [12, 66, 67] and in patients with AIDS who have oropharyngeal candidiasis (OPC) [8, 13, 14].

A great deal of information is now available relating fluconazole MICs determined by NCCLS methods with clinical outcome in patients with AIDS and OPC [8, 12–14]. The published data have been reviewed most recently by Ghanmou et al. [8] and by Rex et al. [14], and it appears that clinical response in OPC correlates well with in vitro susceptibility determinations by NCCLS methods. Unfortunately, there have been relatively few studies attempting to establish a correlation between fluconazole MICs and outcome of candidemia or deep-seated candidiasis, and the data to support an in vitro–in vivo correlation for antifungal susceptibility studies are not as strong as those for OPC [8, 13, 14].

In an effort to establish MIC interpretive breakpoints for fluconazole, the NCCLS Subcommittee on Antifungal Testing has recently reviewed a package of in vitro and clinical data submitted by Pfizer Pharmaceuticals (Groton, CT). Analysis of these data indicates that they are consistent with previously published data [8], and the subcommittee has voted to recommend tentative fluconazole interpretive MIC breakpoints for use in the clinical laboratory [14]. These interpretive breakpoints and the rationale for their development have been reviewed by Rex et al. [14] and will not be discussed further in this document.

**Itraconazole.** Itraconazole is a potent, lipophilic oral triazole that is becoming an important alternative to fluconazole in the treatment of OPC [11, 68, 69]. Thus far, there have been no reports of resistant *C. albicans* strains emerging during the course of itraconazole treatment of OPC [10]; however, studies have demonstrated possible cross-resistance to itraconazole among isolates for which fluconazole MICs were high [70–73] (table 7).

In contrast to the data for fluconazole, there are fewer or no published data directly relating itraconazole MICs determined by the NCCLS method to clinical outcome [8, 14]. The vast majority of clinical isolates of *Candida* species appear highly susceptible to itraconazole: >90% of isolates are inhibited by ≤0.1 μg/mL, and 50%–75% are inhibited by ≤0.12 μg/mL (table 6) [37, 39, 40].

<table>
<thead>
<tr>
<th>Fluconazole group</th>
<th>No. of isolates</th>
<th>Range</th>
<th>MIC₅₀</th>
<th>MIC₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>50</td>
<td>≤0.03–0.5</td>
<td>≤0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>Resistant</td>
<td>50</td>
<td>≤0.03–&gt;16</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

NOTE. Table is adapted from [70].

Although there is some evidence to suggest that itraconazole may be effective in some patients with AIDS who failed to respond to fluconazole [10, 68, 74], the fact that many of these isolates may also have elevated itraconazole MICs of 0.5–1.0 μg/mL suggests that higher doses of itraconazole may be necessary to achieve a clinical response [10, 70–72]. Ultimately, most of these patients will have relapses of infection with strains that are highly resistant to fluconazole and cross-resistant to itraconazole and other azoles [72, 75].

As with fluconazole, the NCCLS Subcommittee on Antifungal Testing has recently examined the correlation between itraconazole susceptibility testing results and clinical response in patients with AIDS and OPC. Pertinent data from clinical trials in the United States have been submitted to the subcommittee by Janssen Pharmaceuticals (Titusville, NJ) and have been analyzed for the establishment of interpretive breakpoints for itraconazole. The findings of preliminary analysis of these data are promising and suggest that the relationship between itraconazole MICs and clinical outcome of OPC is such that it will be possible to propose interpretive breakpoints for this agent as well. The proposed interpretive breakpoints and the process underlying their development are reviewed by Rex et al. [14].

**C. neoformans**

As noted above, standardized antifungal susceptibility testing methods have only recently been optimized for the testing of *C. neoformans* [50, 51]. The microdilution modification of M27 using yeast nitrogen broth appears to work quite well, and this method has allowed Witt et al. [52] to address the ability of in vitro susceptibility testing of fluconazole to predict response to therapy among patients with cryptococcal meningitis.

These investigators studied 76 patients with acute AIDS-associated cryptococcal meningitis who were treated with fluconazole ± flucytosine. They reported that the probability of successful fluconazole treatment could be predicted from the results of in vitro susceptibility testing and blood cultures [52]. They demonstrated that the probability of treatment failure would be ~25% for a patient whose blood is culture-positive for *C. neoformans*, who does not receive flucytosine therapy, and for whose isolate the MIC of fluconazole is 0.25 μg/mL.
This probability of failure increases to >80% if the fluconazole MIC for the isolate is 16 μg/mL [52].

These data suggest that in vitro susceptibility testing results, coupled with additional laboratory and clinical data, may be useful in predicting clinical outcome of cryptococcal meningitis. Although these results are encouraging, additional studies are necessary to confirm the role of susceptibility testing in this setting.

Filamentous Fungi

Rapid progress is being made in developing standardized methods for in vitro susceptibility testing of filamentous fungi [47, 48]. As with the yeasts, it will be necessary to establish in vivo correlates of these in vitro test results. A working group of the NCCLS subcommittee has been established to address this issue and is currently conducting an assessment of antifungal drug efficacy in an animal model using isolates of varying in vitro susceptibility. Findings of preliminary evaluation of these data are promising.

Recommendations for Use of Antifungal Susceptibility Testing in the Clinical Laboratory

Given all the recent developments in the area of medical mycology and antifungal susceptibility testing, the clinical laboratory is faced with an increasing need to set appropriate and relevant policies for implementation and use of the newly available tools. One possible set of guidelines is shown in table 8.

Complete identification of invasive fungal pathogens is the first step with any isolate and is tremendously valuable by itself. For example, knowing the species of Candida permits reasonably reliable prediction of relative antifungal susceptibility (table 6) [54]. With mold fungi, completely identifying the fungus is often the only tool that can be used to help guide therapy and predict outcome.

Despite the many recent advances in this area, antifungal susceptibility testing is not recommended on a routine basis in any setting (table 8), nor will most clinical laboratories want to provide this test. Low-volume laboratories will likely find it difficult to maintain proficiency and would be better off referring the few isolates that require testing to an experienced reference laboratory. As noted above, identification to the species level may be all that is necessary to guide therapeutic decision-making, and this form of testing for the common Candida species should be possible within all but the smallest laboratories [59].

For most laboratories, the most common use of antifungal susceptibility testing will be as a part of periodic batch testing of relevant clinical yeast isolates. Knowledge of the local antibiogram for Candida species vs. fluconazole would be valuable. This approach is similar to that taken with uncommon or difficult-to-test bacteria (e.g., the anaerobes) and may provide data that, when coupled with an accurate identification to the species level, are completely satisfactory for clinical purposes.

Antifungal susceptibility testing of isolates from patients with AIDS and refractory OPC may be useful on occasion. The relevant setting will be that of a patient whose therapy with a moderate or high dose of an azole antifungal is failing (e.g., failure of fluconazole at >200 mg/d). Knowledge that a patient’s therapy is failing despite the fact that the MIC of the infecting organism is low may help uncover problems with compliance, drug absorption, or drug-drug interactions. If deemed appropriate, testing against both fluconazole and itraconazole would be useful.

Our ability to use antifungal susceptibility testing to predict outcome of invasive candidal infections is more limited, but consideration of the MIC in conjunction with the patient’s clinical scenario can be helpful. Testing of Candida species isolates against fluconazole should be offered on request. In the absence of a licensed parenteral formulation of itraconazole, testing for this drug will be needed less often but might be considered in settings where long-term therapy for localized infections is required. Testing against flucytosine might similarly be useful on occasion.

It is important to note that development of resistance during courses of therapy lasting for periods shorter than several weeks

<p>| Table 8. Recommendations for studies of fungal isolates in the clinical laboratory. |</p>
<table>
<thead>
<tr>
<th>Setting</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Routine</td>
<td>Identify all Candida isolates from deep sites to the species level. Identify molds associated with documented infection to the genus level. Identification to the species level is desirable but not absolutely necessary. Routine susceptibility testing is not indicated for any class of isolates.</td>
</tr>
<tr>
<td>Epidemiological survey</td>
<td>Periodic batch antifungal susceptibility testing of isolates obtained from hospitalized patients could be performed to establish an antibiogram for an institution. Relevant species: Candida; relevant drugs: fluconazole, flucytosine.</td>
</tr>
<tr>
<td>AIDS and oropharyngeal candidiasis</td>
<td>Antifungal susceptibility testing may be useful for patients not responding to azole therapy. Relevant drugs: fluconazole, itraconazole. Routine testing not required.</td>
</tr>
<tr>
<td>Invasive candidiasis</td>
<td>Testing of isolates from deep sites, especially non-albicans isolates, may be useful for selected patients. Such testing should be offered upon request. Relevant drugs: fluconazole, itraconazole (rare), flucytosine (rare).</td>
</tr>
<tr>
<td>Cryptococcosis</td>
<td>Neither an optimal testing method nor interpretive guidelines have been established. Testing is not recommended.</td>
</tr>
<tr>
<td>Mold infections</td>
<td>Neither an optimal testing method nor interpretive guidelines have been established. Testing is not recommended.</td>
</tr>
</tbody>
</table>
has not been seen [65]. As a consequence, testing of more than one isolate usually will not be helpful.

Testing of C. neoformans isolates is not yet recommended because of the lack of a clear understanding of optimal methods for testing and interpretation. While limited conclusions can be drawn correlating elevated fluconazole MICs with increased failure rates [52], additional work is required before interpretive breakpoints can be established.

All of the caveats that apply to antifungal susceptibility testing of yeast isolates pertain to testing of mold fungi. Almost nothing is known about relevant testing methods for these fungi, and testing is not recommended. Rather, isolates should be identified to the genus or species level, and published experiences with candidate antifungal agents should be reviewed.

Summary and Conclusions

The field of antifungal susceptibility testing has progressed considerably since 1982. In many ways, the state of the art for susceptibility testing of yeasts is comparable to that of bacteria. The development of standardized susceptibility testing procedures, quality control strains, and user-friendly alternatives to the reference method have placed antifungal susceptibility testing well within the reach of many clinical microbiology laboratories.

Although establishing a correlation between in vitro test results and clinical outcome has been difficult, it is now clear that antifungal susceptibility testing can predict outcome in several clinical situations, the most notable of which is fluconazole treatment of OPC in patients with AIDS. The establishment of tentative interpretive breakpoints for in vitro susceptibility of yeasts to several antifungal agents is now imminent, and efforts are well under way to develop a standardized method for testing of the filamentous fungi.

Despite this progress, it remains to be seen how useful antifungal susceptibility testing will be in guiding therapeutic decision-making. Future efforts must be directed toward establishing and validating interpretive breakpoints for currently available antifungals and toward refining and optimizing procedures for testing amphotericin B, C. neoformans, and molds.

Finally, this entire process must be adapted to include the new antifungal agents currently under development. Each of these new agents will pose additional challenges to the existing methodology, which may require additional adjustments in order to accurately reflect their clinically relevant antifungal activity.

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References

23. Pfaller MA, Grant C, Mortland V, Rhine-Chalberg J. Comparative evaluation of alternative methods for broth dilution susceptibility testing of


