

## FOCUS

# Antifungal Susceptibility Testing: Practical Aspects and Current Challenges

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INTRODUCTION .....	643
METHODS FOR SUSCEPTIBILITY TESTING.....	644
NCCLS Broth-Based Methodology for Yeasts .....	644
Status of the reference method .....	644
Potentially useful modifications of the reference method .....	645
Commercial broth-based MIC systems.....	645
NCCLS Methodology for Molds .....	645
Etest .....	646
Other Agar-Based Testing Methods.....	646
Flow Cytometry and Use of Viability Dyes.....	646
Direct Measurement of Azole Effect: Quantitation of Ergosterol Synthesis .....	646
Other Antifungal Susceptibility Testing Methods for Yeasts and Molds.....	646
Testing of Dermatophytes.....	647
Determination of MFCs rather than MICs .....	647
INTERPRETATION OF RESULTS.....	647
General Principles .....	647
Support for the Clinical Relevance of NCCLS M27 MICs in Yeasts .....	648
Fluconazole and <i>Candida</i> spp.....	648
(i) Pharmacodynamic support for these ideas.....	649
Itraconazole and <i>Candida</i> spp.....	650
(i) Pharmacodynamic support for these ideas.....	650
Ketoconazole and <i>Candida</i> spp .....	650
Amphotericin B and <i>Candida</i> spp.....	651
(i) Pharmacodynamic support for these ideas.....	651
Flucytosine, <i>Candida</i> spp., and <i>C. neoformans</i> .....	651
(i) Pharmacodynamic support for these ideas.....	652
<i>C. neoformans</i> , fluconazole, and amphotericin B.....	652
Molds.....	652
Other Drugs.....	653
CONCLUSIONS .....	653
REFERENCES .....	653

### INTRODUCTION

Antifungal susceptibility testing remains an area of intense interest. Susceptibility testing can be used for drug discovery

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and epidemiology, but this review will focus on use of antifungal susceptibility testing to predict therapeutic outcome. With the demonstration that susceptibility of *Candida* spp. to azole antifungal agents (particularly fluconazole) generated correlations with clinical outcome for some forms of candidiasis that were qualitatively similar to that seen for antibacterial agents (182) and the steady introduction of new drugs of both preexisting and new classes (9), the interest in and need for clinically relevant susceptibility testing has increased. The need extends

TABLE 1. NCCLS conditions for antifungal susceptibility testing

Characteristic	NCCLS M27-A (135)	NCCLS M38-P (133)
Suitability	Yeasts	Conidium- and spore-forming fungi
Inoculum	$0.5 \times 10^3$ – $2.5 \times 10^3$ CFU/ml	$0.4 \times 10^4$ – $5 \times 10^4$ CFU/ml
Inoculum standardization	Spectrophotometric, with reference to a 0.5 McFarland BaSO <sub>4</sub> turbidity standard	Spectrophotometric, with specification of a target optical density that varies by fungal genus
Test medium	RPMI 1640, buffered to pH 7.0 with 0.165 M MOPS	Same as M27-A
Format	Macrodilution or microdilution	Microdilution
Temp	35°C	35°C
Duration of incubation	48 h ( <i>Candida</i> spp.), 72 h ( <i>C. neoformans</i> )	24 h ( <i>Rhizopus</i> spp.), 72 h ( <i>Pseudallescheria boydii</i> ), 48 h (most fungi, including <i>Fusarium</i> spp., <i>Aspergillus</i> spp., and <i>Sporothrix schenckii</i> )
End point	Optically clear well for amphotericin B, ~80% reduction in growth (macrodilution testing with azoles), prominent decrease in turbidity (microdilution testing with flucytosine and the azole antifungals)	Prominent growth reduction from control (~50%) for flucytosine, fluconazole, and azoles; optically clear well for amphotericin B; the T-level revision will propose the use of an optically clear end point for itraconazole and the newer azoles (e.g., voriconazole)
QC isolates and drugs	Two isolates of <i>Candida</i> against amphotericin B, flucytosine, fluconazole, voriconazole, ketoconazole, itraconazole, caspofungin (MK-0991), ravuconazole (BMS 207147), posaconazole (SCH 56592), and anidulafungin (LY303366, V-echinocandin) (14)	Two isolates of <i>Aspergillus</i> against amphotericin B and itraconazole

beyond testing *Candida* spp. With resistance demonstrated among such diverse fungi as *Cryptococcus neoformans* (1, 150, 167, 185, 227), *Aspergillus fumigatus* (40, 47, 96, 104, 130), *Aspergillus terreus* (207, 216), *Histoplasma capsulatum* (224), *Pseudallescheria boydii* (*Scedosporium apiospermum*) (219), and *Trichosporon* spp. (217, 218), it is clear that the need for meaningful susceptibility test results is as great for the fungi as it is for the bacteria.

Although antifungal susceptibility testing remains less well developed and utilized than antibacterial testing, the scientific support for its validity has benefited greatly by extrapolation from antibacterial testing. Knowledge of mechanisms of antifungal resistance has been valuable in identifying resistant isolates and using them to validate in vitro measurement systems (75, 130, 213, 225). The application of the concepts of pharmacodynamics (W. A. Craig, Abstr. 36th Annu. Meet. Infect. Dis. Soc. Am., abstr. S67, 1998) to antifungal susceptibility testing (4–7; 23, 52, 54, 81, 85, 99, 100, 113; D. A. Andes, M. van Ogtrop, T. Stamsted, and B. Conklin, Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998, abstr. F-88, 1998; E. J. Wolfe, M. E. Klepser, and M. A. Pfaller, Abstr. 34th Annu. Meet. Infect. Dis. Soc. Am., abstr. 76, 1996) has provided significant new insights into both the methods for and interpretation of antifungal susceptibility testing. In addition, novel techniques for susceptibility testing provide useful additional tools and insights. It is the purpose of this review to summarize developments in this area since the last major reviews on this topic (59, 67, 74, 160, 183). This paper is not an exhaustive review of all prior work in this area, and it will focus on the issues that are important to testing in the clinical laboratory with special emphasis on the well-studied National Committee for Clinical Laboratory Standards (NCCLS) methodologies. For additional background information, the interested reader is referred to these reviews for additional data.

## METHODS FOR SUSCEPTIBILITY TESTING

### NCCLS Broth-Based Methodology for Yeasts

**Status of the reference method.** After passing through the stages of being a (P)roposed document (M27-P) in 1992 (134) and a (T)entative document (M27-T) in 1995 (136), the NCCLS M27 methodology for testing of yeasts became an (A)pproved level document (M27-A) in 1997 (135). The process by which this method was developed has been extensively reviewed (74, 160, 183). Publication of M27-A was the culmination of approximately 15 years of collaborative work. Although imperfect, this method has the great advantage of high interlaboratory reproducibility. Combined with the secondary knowledge generated during its development concerning factors that both add to and detract from reproducibility, M27 has become a valuable tool for all investigators in this field. The method specifies inoculum size and preparation, test medium, incubation time and temperature, and end-point reading for flucytosine, amphotericin B, fluconazole, ketoconazole, and itraconazole (Table 1). The reference method was initially defined solely as a macrodilution methodology, but this was extended with a microdilution method that was included beginning with the T-level revision of the document. The microdilution method has become the method of choice because of its less cumbersome nature. Microdilution plates may easily be prepared and frozen well in advance of use.

The M27 method continues to be augmented. The M27-A document provides Quality Control (QC) limits at 48 h for amphotericin B, flucytosine, fluconazole, ketoconazole, and itraconazole. QC data were recently expanded to include 24-h QC limits as well for these previously mentioned agents (14). In addition, QC limits at 24 and 48 h for voriconazole (UK-109,496), caspofungin (MK-0991), ravuconazole (BMS 207147), posaconazole (SCH 56592), and anidulafungin (LY303366, V-echinocandin) have been provided (14). How-

ever, and as noted below, interpretive breakpoints have been established for fluconazole, itraconazole, and flucytosine against *Candida* spp. only after 48 h of incubation.

The M27-A methodology for *Candida* recommends an end-point reading at 48 h (Table 1). For most isolates, the difference between reading at 24 and 48 h is minimal and will not alter the interpretative category (i.e., does not change whether the isolate would be categorized as susceptible or resistant). However, recent work has begun to include 24-h readings because (i) MICs can often be read at 24 h and (ii) readings taken at 24 h may be more clinically relevant for some isolates. Isolates for which the earlier reading is important show a dramatic rise in drug MIC between 24 and 48 h due to significant trailing growth (partial inhibition of growth over an extended range of antifungal concentrations). Estimated as occurring in about 5% of isolates (12), this trailing growth can be so great as to make an isolate that appears susceptible after 24 h appear completely resistant at 48 h. Two independent *in vivo* investigations of this phenomenon that employed murine models of disseminated candidiasis (12, 181) have shown that isolates with this behavior should be categorized as susceptible rather than resistant. This concept has been corroborated by a demonstration that trailing growth can be eliminated by lowering the pH of the test medium to 5 or less (122) and by a clinical demonstration that oropharyngeal candidiasis due to such isolates responds to a low dose of fluconazole used to treat typical susceptible isolates (179).

The MIC end point is also of major importance, especially for isolates exhibiting trailing growth. The end point for azoles for both macro- and microdilution testing has been defined at the point at which there is prominent reduction in growth. For macrodilution testing, prominent reduction in growth was shown to be equivalent to the turbidity of a 1:5 dilution of the growth control (63). This end point could also be referred to as 80% reduction in growth relative to the growth control. However, when the microdilution format is utilized and read spectrophotometrically, the 80% rule is overly restrictive and 50% inhibition of growth by spectrophotometry best approximates the visual endpoint (55, 145, 156, 205).

**Potentially useful modifications of the reference method.** Although not included in the reference NCCLS methodology, supplementation of the RPMI 1640 incubation medium with 18 g of glucose per liter (to change the final glucose concentration from 2 to 20 g/liter) has been shown repeatedly to enhance isolate growth without significantly altering the observed MICs of amphotericin B, flucytosine, ketoconazole and fluconazole (25, 38, 45, 66, 189, 190). Agitation of microdilution plates prior to reading is required for spectrophotometric readings and appears to simplify visual reading of end points (3). Agitation is also very useful for reading end points with *Cryptococcus neoformans*, since this yeast tends to form a button-like deposit in the microdilution wells (73).

Another modification that might be of value is reducing the molarity of the morpholinepropanesulfonic acid (MOPS) buffer. Tornatore et al. demonstrated that reduction of MOPS concentration from 0.165 to 0.0165 M lowered the frequency with which the NCCLS methodology overcalled resistance in a selected set of *C. albicans* isolates (211). Shortening the incubation period to 24 h (181) and reducing the medium pH below 5 (122) have also been proposed as methods to clarify

trailing end points for *Candida* spp. In addition, continuous agitation of the microdilution plate may enhance the growth of *C. neoformans* (188). However, none of these modifications have achieved broad acceptance.

**Commercial broth-based MIC systems.** Recently studied broth-based commercial systems include Candifast (International Microbio/Stago Group, Milan, Italy), Integral Systems Yeasts (Liofilchem Diagnostics, L'Aquila, Italy), and Fungitest (Bio-Rad SDP [formerly Sanofi Diagnostics Pasteur], Paris, France). These kits provide reagents for testing a limited number of drug concentrations selected as critical breakpoint values. Direct comparisons have found these methods to possess limited correlation with the M27-A reference method (42, 165, 196, 208, 226, 229). ATB Fungus (API-bioMerieux, Marcy l'Etoile, France), Mycostandard (Institut Pasteur, Paris, France), and Mycototal (Behring Diagnostic, Rueil-Malmaison, France) are similarly designed systems for which only limited data are available on correlation with the M27-A methodology (51, 84, 174, 196).

A commercial system that includes a colorimetric response based on the Alamar Blue redox marker is marketed as Sensititre Yeast One Colorimetric Antifungal Panel (Trek Diagnostic Systems, Inc., Westlake, Ohio). This kit tests a full range of drug concentrations, and rates of concordance of  $\geq 85\%$  with the reference method are generally obtained (43, 65, 112, 157, 165, 172), although one group noted some difficulties with testing of *C. neoformans* (112). The ASTY colorimetric panel (Kyokuyo Pharmaceutical Industrial, Ltd., Tokyo, Japan) demonstrated a similarly good correlation with the M27 reference method (153). Some of these kits are currently being evaluated by the U.S. Food and Drug Administration; however, to date none have received approval for marketing in the United States.

### NCCLS Methodology for Molds

Direct adaptations of the M27-A methodology to *Aspergillus* spp., *Chaetomium* spp., *Cladophialophora* spp., *Fusarium* spp., *Pseudallescheria boydii*, *Rhizopus arrhizus*, and *Sporothrix schenckii* were shown to generate reproducible results (62, 170, 171). Following the principles established for testing yeasts, a proposed standard method entitled "Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi" has now been published as NCCLS M38-P (133). This method was developed using isolates of *Aspergillus* spp., *Fusarium* spp., *Rhizopus* spp., *P. boydii*, and *S. schenckii*. Recent data suggest that *Bipolaris* spp., *Cladophialophora* spp., *Dactylaria* spp., *Paecilomyces* spp., *Scedosporium* spp., *Trichoderma* spp., and *Wangiella dermatitidis* can be tested using this method (A. Espinel-Ingroff, V. Chatuvedi, A. Doney, A. Fothergill, and M. Rinaldi, Program Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-2, 2000). Other fungi have not been specifically studied, nor is there a methodology that addresses the issue of fungi which do not produce spores. The methods of inoculum preparation, choice of inoculum size, time of reading, and end-point selection have all been evaluated in a series of collaborative studies (60, 62). Nongerminated conidia are used since, at least for *Aspergillus* spp., similar results are obtained for germinated and nongerminated conidia (18, 56, 120). Different results

might be obtained with other species (83), and this area may require further study. A significant difference from M27-A is the inoculum, which is approximately 10-fold higher than for yeasts and requires a different method of preparation (Table 1). Due to the differences in size and light-scattering properties of many kinds of spores that can be generated by these fungi, the M38 method specifies different optical density ranges for each genus. Careful preparation of the inoculum is important, since a concentration outside the specified range will result in an elevated MIC to most antifungal agents (72, 130).

The end-point definition is another area of difference for M27 and M38. In M27, azoles are read at a partial inhibition end point (defined as the lowest drug concentration producing a prominent reduction in growth). While this wording is used in the M38-P document for the azoles, more recent data suggests that reading the end point at 100% inhibition (no growth) better detects resistance of *Aspergillus* spp. to itraconazole and the newer triazoles (45; A. Espinel-Ingroff, Program Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 154, 1999). This modification has been proposed for the T-level revision of the M38 document.

### Etest

Etest (AB Biodisk, Solna, Sweden) is a proprietary, commercially available method for antimicrobial susceptibility testing. MICs are determined from the point of intersection of a growth inhibition zone with a calibrated strip impregnated with a gradient of antimicrobial concentration and placed on an agar plate lawned with the microbial isolate under test. This methodology has been adapted to a number of antifungal agents. Both nonuniform growth of the fungal lawn and the frequent presence of a feathered or trailing growth edge can make end-point determination difficult. However, with experience and standardized techniques, the correlation between this method and the reference method has been acceptable for most *Candida* spp. and the azole antifungal agents (30, 35, 64, 68, 155, 158, 165, 199, 221, 222). One study, however, did observe less than 50% concordance between the methods for *C. tropicalis* and *C. glabrata* with fluconazole (199), and another found a limited correlation for ketoconazole and *C. lusitaniae* (68). These results suggest that users of the Etest methodology should carefully validate their local procedures against the reference methodology. For many molds, including *Aspergillus* spp., good correlations with amphotericin B and itraconazole Etest and MICs by the M38 method have been demonstrated (159, 209). Etest has significant value for the determination of amphotericin B MICs and represents one of the more reliable ways to identify resistant isolates (see below). The choice of growth medium appears critical with this technique, with RPMI-based agars generally appearing most useful (e.g., this agar gave >96% correlation with the reference microdilution method in one study [159]). QC Etest limits for the two NCCLS M27 QC isolates (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019) against amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole have been proposed (155).

### Other Agar-Based Testing Methods

Disk-based susceptibility testing is convenient and economical. Adaptations with good correlation with the reference broth methods have been shown for fluconazole (13, 125, 194). Flooding the surface of the plate with methylene blue (0.5  $\mu\text{g/ml}$ ) appears to improve zone edge definition and facilitates reading (165). A commercial disk system (Neo-Sensitabs; Rosco Diagnostica, Taastrup, Denmark) is available, but the results were not found to correlate well with the reference method (25, 202, 208, 226). Reports on the Diff Test (Diagnostics Pasteur, Paris, France) disk diffusion system and a spiral gradient method did not include comparisons with the reference method (51, 196).

### Flow Cytometry and Use of Viability Dyes

Flow cytometry has been long recognized as a possible tool for antifungal susceptibility testing and has been developed for yeasts in studies that focused principally on *Candida* spp. (2, 69, 79, 146, 161–164). Staining (or lack of staining) with suitable dyes permits the rapid detection of damaged fungi. Recent work has further explored this possibility (80, 88, 97, 151, 175, 176, 223) and shown the potential for correlation of flow cytometry-based techniques with the reference method. One study suggested that flow cytometry might be especially useful for detection of amphotericin B resistance (69). In conceptually related studies, fluorescent viability dyes have been used to examine the nature of drug-induced damage to yeasts and moulds (50, 111) and to estimate MICs and minimal fungicidal concentrations (MFCs) for molds (105).

### Direct Measurement of Azole Effect: Quantitation of Ergosterol Synthesis

Given that azole antifungal agents act by inhibition of ergosterol synthesis, direct measurement of alterations in ergosterol synthesis appears relevant. Arthington-Skaggs et al. have described a workable laboratory method for such testing (11) and have shown that it correlates well with the NCCLS M27-A methodology for *Candida* spp. Importantly, both in vitro (11) and in vivo (12) testing of isolates exhibiting trailing growth further corroborates the notion that these isolates should be classified as susceptible, rather than resistant.

### Other Antifungal Susceptibility Testing Methods for Yeasts and Molds

Many alternative methods for antifungal susceptibility testing have been proposed. Shimokawa and Nakayama have shown that testing azoles against *Candida* spp. on acetate-supplemented media produces MICs that more closely approximate the critical concentration at which ergosterol biosynthesis is inhibited (200, 201). Incorporation of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] or XTT (2,3-bis(2-methoxy-4-nitro-5-dulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) as a colorimetric marker for redox potential has been found convenient for *Candida* spp. (33, 86, 91, 210, 231), *C. neoformans* (33, 86), and molds such as *Aspergillus* or *Fusarium* spp. (91, 92, 126; C. C. Chiou, N. Mavrogiorgos, and T. J. Walsh, Program Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 940, 2000; T. J.

Walsh, A. Field-Ridely, D. Sutton, V. Petraitis, R. Petraitiene, T. Sein, R. Schaufele, M. J. Rinaldi, M. Ghannoum, and C. A. Lyman, Abstr. 14th Congr. Int. Soc. Hum. Anim. Mycol., abstr. 557, 2000). This approach generates MICs comparable to those in the NCCLS reference method and presents substantial opportunities for automation. Testing with XTT eliminates an extraction step that is needed with MTT-based assays (86, 210). Similarly, methods for measurement of intracellular ATP concentration (102) and consumption of glucose from the incubation medium (184) have yielded excellent correlation with the reference method. Measurement of medium capacitance provides a very early assessment of fungal growth and could permit the estimation of an MIC directly from the initial positive culture specimen (e.g., from the material in the blood culture bottle) without the need for subculture (29). Spectrophotometric measurement of incorporation of neutral red by viable cells was shown to correlate with an estimate of the MFC for *Trichophyton* spp. (71). Radiometric and direct microscopic methods have also been described in the past (183) but are cumbersome and little used in recent work.

Agar-based methods are attractive due to their simplicity. An agar dilution-based method incorporating a specific concentration of fluconazole (8  $\mu\text{g/ml}$ ), in combination with the chromogenic CHROMagar *Candida* medium, is a useful screening tool for fluconazole resistance (147, 148). This method, however, still requires formal testing of putatively resistant isolates. An agar dilution method based on semisolid agar has also been reported to render significant concordance with the reference method (169). Incubation under 20%  $\text{CO}_2$  has been suggested as a way to clarify the end point in an agar dilution method (232).

### Testing of Dermatophytes

Dermatophytes grow slowly, and therefore agar-based methods have often been employed (24, 118, 214). Most studies, however, have focused on drug comparisons and employed methods that are not standardized or correlated with clinical outcomes. Thus, the clinical relevance of in vitro testing for the dermatophytes remains unclear. However, multiple efforts are under way to develop standardized methods for these fungi (95, 142; K. C. Hazen, A. E. Moon, and E. S. Atillasoy, Program Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 204, 2000). Much of the initial work has focused on the selection of medium, with particular attention to choosing a medium that supports conidial formation in *Trichophyton rubrum*. Subsequent work investigating the inoculum and end point is under way.

### Determination of MFCs rather than MICs

MFCs have the potential for being more relevant to clinical outcome, especially in the context of profoundly immunocompromised hosts or body sites (e.g., heart valves) where host defenses are at a disadvantage. Indeed, some work with MFCs has suggested that they may be more predictive than MICs even for general testing (137, 186, 217, 218). However, all of the issues of standardization that occur with MICs also apply to MFCs. And, new issues arise including questions of drug carryover, sample volume, and end point. Even the very definitions of the terms "microbicidal" and "microbistatic" come

into question—a bactericidal antibacterial drug kills  $\geq 99.9\%$  of organisms, but should such a definition apply to fungi with sixfold-longer doubling times (e.g., 20 min versus 2 h)? Nevertheless, and extrapolating from standardized testing for measurement of bactericidal activity (132), Klepser et al. have approached MFCs by measuring time-kill curves for antifungal agents (98). Use of time-kill curves has been relevant to their work on antifungal pharmacodynamics (see below) and provides additional support for the validity of any chosen measurement. Methods that would reliably generate such a curve could be directly applied to the measurement of MFCs at any given time point. Klepser has proposed a standardized method (98) and made extensive use of this technique in a series of pharmacodynamic investigations (52, 54, 85, 99, 100, 110; Wolfe et al., 34th Annu. Meet. IDSA). Killing tests are being further investigated in a collaborative fashion, and these methods would appear to represent an excellent starting point for further research in this area.

## INTERPRETATION OF RESULTS

### General Principles

The ability to generate an MIC is of little value without the corresponding ability to interpret its clinical meaning. However, this process is far from straightforward because (i) MICs are not a physical measurement, (ii) host factors play a critical role in determining clinical outcome, (iii) susceptibility in vitro does not uniformly predict clinical success in vivo, and (iv) resistance in vitro will often, but not always, correlate with treatment failure (182). The initial publication of the M27 document included interpretive breakpoints for *Candida* spp. when tested against fluconazole, itraconazole, and flucytosine (Table 2). The data for each drug had different strengths and weaknesses for determining these interpretive breakpoints, as discussed below.

A weakness that applies to all of the MIC-clinical outcome correlation data sets is the relative paucity of clinical outcome data for isolates for which the MICs are elevated. Such potentially resistant isolates are infrequently encountered in most early clinical trials, and this circumstance limits the strength of proposed breakpoints. A second limitation, unique to antifungal drugs, is that all (itraconazole) or most (fluconazole) of the data were derived from studies of mucosal candidiasis. There are fewer data for invasive candidiasis. Despite these limitations, the general level of clinical correlation achieved to date is similar to that seen with antibacterial agents.

While quality outcome data relating a range of drug doses to treatment results for infections due to isolates for which the MICs are elevated are not likely to be forthcoming, there are other means of providing additional support in this area. In particular, lessons can be learned from applying antifungal susceptibility testing to the field of time-kill studies and pharmacodynamics that may have direct clinical applications. This area has been well developed for antibacterial agents, and excellent reviews are available (36, 37, 131). In brief, pharmacodynamic analyses attempt to integrate both blood level and MIC into the prediction of clinical outcome. Although interrelated, one of three possible parameters tend to produce the best correlation for any given drug: (i) the proportion of the

TABLE 2. Meaningful test methods and conditions for antifungal susceptibility testing

Organism	Drug	Methods	Interpretation	References
<i>Candida</i> spp.	Fluconazole	M27-A and methods that give concordant results	S, <8 µg/ml; S-DD, >8 µg/ml and ≤32 µg/ml; R, >32 µg/ml; data are best supported for mucosal disease, but the general principles also appear to hold for invasive disease; adequate drug dose is critical as the MIC rises; <i>C. krusei</i> is assumed intrinsically resistant and should not be tested	32, 108, 135, 182
	Itraconazole	M27-A and methods that give concordant results	S, ≤0.125 µg/ml; S-DD; >0.125 µg/ml and ≤0.5 µg/ml; R, >0.5 µg/ml, data apply only to mucosal disease; ensuring adequate absorption by use of the solution is often critical to drug efficacy	135, 182
	Ketoconazole	M27-A-like methods	No specific breakpoints proposed, but aggregate data suggest that isolates with an MIC of >0.125 µg/ml by M27-A are less likely to respond	27, 123, 128, 191, 205
	Flucytosine	M27-A-like methods	S, ≤4 µg/ml; I, >4 µg/ml and ≤16 µg/ml; R, >16 µg/ml; these breakpoints are based largely on historical data and animal models but appear rational based on the available clinical and pharmacokinetic data	7, 70, 89, 135
	Amphotericin B	M27-A-like methods that use AM3 broth, Etest, or MFC methods	Significant controversy and difficulty; agar-based methods appear to have the most potential, in general, and isolates with amphotericin B MICs of >0.5 µg/ml are probably resistant; specific breakpoints have NOT been proposed, and intralaboratory variation makes use of reference isolates with known resistance mandatory; fortunately, resistance appears uncommon among the four most common species (106)	34, 137, 180, 221
<i>C. neoformans</i>	Fluconazole	Modified M27-A (YNB broth, higher inoculum)	No specific breakpoints proposed; Data are limited but consistent: MICs of ≥2–4 µg/ml by this method appear to predict a greater chance of failure; failure definitely appears more likely as the MIC reaches 16 µg/ml	1, 93; Witt et al., abstr. <sup>a</sup>
	Flucytosine	M27-A-like methods	No specific breakpoints proposed, but breakpoints similar to those for <i>Candida</i> spp. (see above) would appear rational	
	Amphotericin B	M27-A-like methods that use AM3 broth, Etest	Significant controversy and difficulty; agar-based methods have the most potential in general. Measurement of fungicidal concentrations may have value; no specific breakpoints proposed—testing should incorporate reference isolates with known resistance.	117, 185, 186
Mold fungi	Any	Unknown	M38-like methods correlate for itraconazole and <i>A. fumigatus</i> ; correlations for amphotericin B are ill-defined	See text

<sup>a</sup> M. D. Witt, L. A. Mortara, R. A. Larsen, R. J. Lewis, J. E. Edwards, and M. Ghannoun, Abstr. IXth Int. Conf. AIDS, abstr. WS-B12-4, 1993.

time that the drug's concentration exceeds its MIC for the organism, (ii) the ratio of the maximum concentration of the drug to its MIC for the organism ( $C_{\max}/\text{MIC}$ ), and (iii) the ratio of the drug's area under the exposure curve to its MIC for the organism (AUC/MIC). Data from relevant pharmacodynamic studies will be introduced as each drug is discussed in turn, below.

On a more practical note, inter-run and interlaboratory variation is a definite limitation for all susceptibility testing methodologies. It must be clearly understood that a single measurement of a MIC is at best an estimate. Inter-run and interlaboratory variability consistently lead to the observation that repeated testing for any given isolate produces MIC that usually span no less than three twofold dilutions. This is often seen in collaborative studies used to generate QC limits (14). Even when highly experienced laboratories use common materials to perform testing, isolates which generate MICs only within a span of three twofold dilutions are the exception rather than the rule. And, if great care is not taken with all the technical details (177), it is unfortunately easy to generate erroneous results. Thus, prediction of a therapeutic outcome

from an isolate for which the MIC is at the boundary between susceptible and resistant should always be undertaken with great care!

#### Support for the Clinical Relevance of NCCLS M27 MICs in Yeasts

**Fluconazole and *Candida* spp.** The M27-A method includes interpretive breakpoints for *Candida* spp. tested against fluconazole (182). These breakpoints were based on an analysis of treatment outcomes in both mucosal and invasive disease and included the description of a novel end point named S-DD, (susceptibility is dose dependent). For fluconazole, the S-DD range includes MICs of 16 and 32 µg/ml. Isolates with fluconazole MICs above this range are classified as resistant (R), whereas those inhibited at lower concentrations are simply labeled susceptible (S). The S-DD breakpoint concept emphasizes the importance of attaining maximal fluconazole levels in blood and tissue for isolates with higher MICs. Maximal dose is defined in this context as being at least 400 mg/day in a 70-kg adult with normal renal function. The weaknesses of these data

TABLE 3. Dose/MIC ratios from the NCCLS M27 fluconazole correlation data set<sup>a</sup>

Dose/MIC	48-h MIC		24-h MICs	
	No. cured/total no.	% Cure (95% CI)	No. cured/total no.	% Cure (95% CI)
>800	26/27	96 (81–100)	162/165	98 (95–100)
400–600	89/89	100 (96–100)	106/106	100 (97–100)
100–300	129/132	98 (94–100)	66/69	96 (88–99)
50–75	34/37	92 (78–98)	36/39	92 (79–98)
25–37.5	30/33	91 (76–98)	25/32	78 (60–91)
12.5–6.25	35/47	74 (60–86)	28/43	65 (49–79)
<6.25	30/46	65 (50–79)	12/21	57 (34–78)

<sup>a</sup> The original data set presented as the support for the NCCLS M27 breakpoints (182) was reanalyzed using dose/MIC as the grouping factor. MICs were obtained by the M27-A macrodilution methodology, and the analysis was limited to patients with oropharyngeal candidiasis. The analysis is also shown for isolates wherein a 24-h MIC was reported using the M27-A methodology. For each percentage cure or aggregate percentage cure, a 95% confidence interval (CI) (233) is also shown in parentheses.

were that (i) two-thirds of the results were drawn from cases of mucosal candidiasis and (ii) the concept of dose-dependent susceptibility was most clearly proven only for mucosal candidiasis. An additional concern is that children have significantly different pharmacokinetic parameters for fluconazole than adults (20, 107, 195, 197), and none of the data have addressed in vitro-in vivo correlations in this population.

These breakpoints have been supported by additional clinical data on oropharyngeal candidiasis in recent publications. Using the Etest method, Dannaoui et al. reported that infection due to *C. albicans* isolates with a fluconazole MIC of  $\geq 48$   $\mu\text{g/ml}$  was likely to be unresponsive to fluconazole at median doses of 100 to 200 mg/day (41). Using a non-M27 broth-based method, Cartledge et al. reported that infections due to isolates with fluconazole MICs of  $>8$   $\mu\text{g/ml}$  were unlikely to respond to 100 mg of fluconazole per day (27). Quereda reported that infections due to isolates with fluconazole MICs of  $\geq 8$   $\mu\text{g/ml}$  (by M27-A, but in medium supplemented to 20 g of glucose per liter) were less likely to respond to 100 mg of fluconazole per day (173). Using doses of fluconazole that increased from 100 mg/day to as high as 800 mg/day as needed, Revankar et al. reported that clinical failure only occurred when the M27-A MIC was  $\geq 64$   $\mu\text{g/ml}$  (178). This study clearly documented the need for increased doses of fluconazole as the fluconazole MIC rose. In addition, Revankar et al. observed that a longer duration of therapy was required to achieve control over infections due to less susceptible isolates.

In one of the two data sets available for nonmucosal candidiasis, Lee et al. reported outcome in 32 fluconazole-treated (400 mg/day) non-AIDS patients with deep-seated candidal infections (108). *C. albicans* was the most common species in this series, but all the major species were represented. Success rates were 79% (19 of 24 patients infected with S isolates), 66% (4 of 6 patients infected with S-DD isolates), and 0% (0 of 2 patients infected with R isolates). Clancy et al. reported on 34 candidal bloodstream infections treated during very early work with 100 to 200 mg of fluconazole per day. All the major *Candida* spp. were represented, and failure rates were 48, 86, and 100% for isolates testing in the S, S-DD, and R ranges, respectively (C. J. Clancy, C. A. Kauffman, A. Morris, M. L. Nguyen, D. C. Tanner, D. R. Snyderman, V. L. Yu, and M. H. Nguyen, Abstr. 36th Annu. Meet. Infect. Dis. Soc. Am., abstr. 98, 1998). This data set is particularly helpful in that it is one of the few where infections due to isolates for which the MICs

were higher were treated with doses of fluconazole that were (by current standards) low. The doses used would not have been predicted to be adequate for isolates with fluconazole MICs in the S-DD range, and the response of these isolates would be expected to be similar to that of the R isolates. This data set thus provides helpful support for the importance of appropriate dosing in cases of invasive infection.

**(i) Pharmacodynamic support for these ideas.** Pharmacodynamic issues have been most extensively studied for fluconazole. In vitro work suggested that the effect of fluconazole is concentration independent and is maximal when the concentration is two- to eightfold greater than the MIC (23, 99). Response might thus be expected to correlate best with time above the MIC (as for the  $\beta$ -lactam antibiotics) (99). However, and perhaps due to sub-MIC effects on growth, in vivo work has shown that the AUC/MIC ratio is the best predictor of response. The two independent studies in this area examined isolates with fluconazole MICs ranging from 0.5 to 32  $\mu\text{g/ml}$  and concluded that the 50% effective dose  $\text{ED}_{50}$  for in vivo studies was achieved when the AUC/MIC ratio exceeded 12 to 24 (6, 113).

The consistency of the pharmacokinetics of fluconazole permits an instructive calculation to be done from these data. It is known that the 24-h AUC for healthy adults given fluconazole is almost exactly equal to the daily dose in milligrams. That is, a 70-kg adult given 400 mg of fluconazole will have an AUC of 400  $\text{mg} \cdot \text{h/liter}$  (114). The validity of this assumption for patient groups depends, of course, on the likelihood that they would have both normal renal function and approximately normal body habitus. The data set from which the M27 interpretive breakpoints for fluconazole were derived contains the dose and MIC for each patient. Body habitus and serum creatinine levels are not available. However, for patients with systemic infection, it is known that the study protocols all specified a fluconazole dose of 400 mg/day. Thus, any variation in dose is likely to have been due to issues of renal function or body habitus.

However, the patients receiving therapy for oropharyngeal disease were generally treated at 100 mg/day and were outpatients, who were likely to have had relatively normal renal function. Dose increases were therefore more likely to have occurred in instances of poor response. In this subset of patients, it is thus of interest to compare outcome with the dose/MIC ratio on the assumption that this approximates the AUC/

MIC ratio (Table 3). The data show a clear relationship between the dose/MIC ratio and clinical outcome. Using 48-h MICs, a clear decrement in response is seen when the dose/MIC ratio falls below 25. Given the well-known tendency for MICs to rise 1 dilution between 24 and 48 h of incubation, it is not surprising that the critical ratio shifts a corresponding amount between 24 and 48 h. Looking at 48-h MICs and the critical AUC (dose)/MIC ratio of 25, this translates to needing 800 mg/day to treat an isolate with a fluconazole MIC of 32  $\mu\text{g/ml}$  and 100 mg/day for an isolate with a fluconazole MIC of 4  $\mu\text{g/ml}$ . The relationships between these values and the NCCLS interpretive categories S, S-DD, and R are apparent.

Also relevant to this discussion, Graninger et al. (78) describe increased rates of successful therapy of candidemia due to *C. albicans* when the dose was increased from 400 to 800 mg/day in two sequential cohorts of  $\sim 30$  patients each. All isolates had a similar low fluconazole MIC (MIC for 90% of isolates [MIC<sub>90</sub>] by an NCCLS M27-like method of 1.25  $\mu\text{g/ml}$ ), and other factors might have been in play. However, this increase in success might also have reflected the consequences of an increased AUC/MIC ratio, and these results are at least consistent with the data reported by Lee et al. (108) and Clancy et al. (36th Annu. Meet. IDSA).

**Itraconazole and *Candida* spp.** For itraconazole, M27-A provided interpretive breakpoints only for mucosal candidal infections. Isolates with an itraconazole MIC of  $\leq 0.125$   $\mu\text{g/ml}$  were classified as S, those with an itraconazole MIC of  $> 0.5$   $\mu\text{g/ml}$  were classified as R, and those in between were classified as S-DD, where the implication is that susceptibility depends on drug delivery. Unlike the case with fluconazole, where its reliable absorption means that the administered dose is virtually always well delivered to the site of infection, itraconazole's bioavailability is unpredictable. The data supplied with the NCCLS itraconazole data set included drug levels in blood, and infections due to isolates in the S-DD range showed a trend toward a better response with higher drug levels (182). The critical level in blood was thought to be approximately 0.5  $\mu\text{g/ml}$  on the basis of high-pressure liquid chromatography. The method of blood measurement is important, since bioactive itraconazole metabolites cause bioassay-based measurement systems to render higher levels in blood (90). This concept is consistent with observations that achieving approximately this level in blood as determined by high-pressure liquid chromatography predicts a greater likelihood of efficacy in the treatment of both experimental (16) and human (76; A. Glas-macher, C. Hahn, E. Molitor, T. Sauerbruch, G. Marklein, and I. G. H. Schmidt-Wolf, Program Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 700, 2000).

Few supportive data have emerged with respect to itraconazole interpretive breakpoints for *Candida* species. Cartledge et al. reported that AIDS patients with oropharyngeal candidiasis treated with 200-mg itraconazole capsules (the least well absorbed formulation of the drug) had a greater rate of failure when the MIC was  $> 2$   $\mu\text{g/ml}$  (27). In a supportive laboratory study, isolates with elevated fluconazole MICs were found to have elevated itraconazole MICs that were usually  $\geq 0.5$   $\mu\text{g/ml}$ , in contrast to  $\leq 0.125$   $\mu\text{g/ml}$  for isolates with lower fluconazole MICs (123).

In contrast to these results, a study of itraconazole oral solution as therapy for fluconazole-refractory oropharyngeal

candidiasis in human immunodeficiency virus (HIV)-infected patients with oropharyngeal candidiasis found no meaningful correlation of itraconazole MICs by M27-A and outcome in a group of 74 patients (193). Itraconazole levels in blood were not monitored, but the use of the oral solution should have produced adequate levels in most patients. Lack of effective antiretroviral therapy in this group of patients may have limited the possible efficacy of any form of therapy, but the lack of correlation is otherwise unexplained. It is also possible that administration of oral itraconazole solution in these patients amounts, at least partially, to topical therapy for oral *Candida* infections, with the direct effects of contact between drug and mucosa complicating any relationship between levels in blood and mucosal disease. However, the itraconazole oral solution was also used in the experiments collecting the data that contributed to the NCCLS correlation data set (182). In aggregate, the data on correlation between therapeutic outcome and itraconazole MIC are somewhat confusing, and this area would benefit from additional work.

**(i) Pharmacodynamic support for these ideas.** In vivo pharmacodynamic data are scant for itraconazole. Using time-kill studies, Burgess et al. found that an isolate of *C. albicans* with itraconazole MICs in the NCCLS M27-A susceptible range responded to itraconazole, with maximal response seen when the drug concentration was double the MIC (23). Interestingly, isolates with itraconazole MICs in the S-DD and R range both failed to respond to itraconazole (23). The authors conclude that isolates with itraconazole MICs in the S-DD range may be less responsive. Of interest, a dose-dependent pharmacodynamic relationship was observed in an experimental model of treatment of invasive aspergillosis with itraconazole (16). This area also requires further study.

**Ketoconazole and *Candida* spp.** Interpretive criteria have not been proposed for ketoconazole MICs. Cartledge et al. reported two correlations of laboratory results with therapeutic outcome in a group of HIV-infected individuals with oropharyngeal candidiasis treated with ketoconazole at 200 mg twice a day. First, testing of relative growth inhibition by a fixed concentration of ketoconazole (0.05  $\mu\text{g/ml}$ ) in a broth-based system ( $10^4$  CFU/ml of inoculum, pancreatic casein digest [CYG] broth, 72-h incubation at 37°C, spectrophotometric reading) was found to correlate with clinical outcome (26). Failure to achieve at least 25% reduction in growth relative to the growth control predicted a high likelihood of failure. In a study using the reference method to generate MICs, a ketoconazole MIC of  $> 0.125$   $\mu\text{g/ml}$  correlated with increased clinical failure (27).

These broth-based observations have been supported by other work using the reference method (sometimes with the RPMI 1640 medium supplemented to 20 g of glucose per liter) (123, 128, 191, 205). Two of these reports provide clinical data on ketoconazole-treated patients with oropharyngeal candidiasis. One report found no clinical failures in a group of isolates with a ketoconazole MIC of  $< 0.25$   $\mu\text{g/ml}$  (205), whereas results from the other study suggested that clinical resistance would be seen if the MIC was  $\geq 0.06$   $\mu\text{g/ml}$  (191). The amount of data on patients infected with isolates for which the MICs were higher were limited in these reports, thus limiting the strength of these conclusions. However, in a supportive analysis, Milan et al. reported that isolates with ketoconazole MICs

of  $\geq 0.25$   $\mu\text{g/ml}$  often had elevated fluconazole MICs. Likewise, Martinez-Suarez and Rodriguez-Tudela reported that isolates with elevated fluconazole MICs usually had ketoconazole MICs of  $\geq 0.06$   $\mu\text{g/ml}$  (123). The correlation with increased fluconazole MIC is relevant because the mechanisms of azole resistance are common across the azoles, and azole MICs tend to rise in parallel (225). Collectively, these observations support the idea that relevant resistance to ketoconazole begins at an MIC of  $\sim 0.125$   $\mu\text{g/ml}$ . Pharmacodynamic data are not available.

**Amphotericin B and *Candida* spp.** Establishing a clear correlation between the amphotericin B MIC and outcome has been difficult. In vitro and in vivo resistance clearly exists (21, 48, 127, 139, 141, 168, 198, 203, 204, 220) and in vivo-in vitro correlations are possible (143). After the M27 methodology was developed, it became clear that its ability to detect amphotericin B-resistant isolates was limited (180). Isolates with proven resistance would have a higher modal or geometric mean MIC on repeated testing, but the extent of MIC differences between resistant and susceptible isolates was too narrow to permit separation of resistant isolates by the type of single-run testing that is normally performed in a clinical laboratory setting. Modifying M27 by substitution of Antibiotic Medium 3 (also known as Penassay broth) supplemented with glucose to 20 g/liter and buffered to pH 7.0 with 0.1 M phosphate and reading the MIC after only 24 h partially alleviated this problem (116, 180, 221). Subsequent work with the colorimetric Alamar Blue marker found that it did not improve the detection of resistant isolates (115).

However, this research also revealed the importance of as yet unidentified technical factors. Looking first at the reports from one of our laboratories (J.H.R.), work with an initial (but rather old) lot of Antibiotic Medium 3 suggested that resistant isolates had an amphotericin B MIC of  $\geq 1$   $\mu\text{g/ml}$  (180). Subsequent work with three newer lots yielded similar results, except that now resistant isolates had an amphotericin B MIC of  $\geq 0.25$   $\mu\text{g/ml}$  (116). This procedure yielded comparable results in a report from another laboratory (106). However, results from a third laboratory found that this approach did not yield an acceptable correlation with clinical outcome when used to test a series of isolates from candidemic patients treated with amphotericin B. Rather, reading at 48 h and without glucose supplementation was preferred, and optimal results were actually obtained if an MFC was determined (M. H. Nguyen, C. J. Clancy, Y. C. Yu, V. L. Yu, A. J. Morris, D. R. Snyderman, D. A. Sutton, and M. G. Rinaldi, Program Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-81, 1997). The critical values were an MIC of  $\geq 1$   $\mu\text{g/ml}$  or an MFC of  $>1$   $\mu\text{g/ml}$ . The cause(s) of these differences in results is unknown.

Agar-based testing generally seems to produce a broader separation of high and low amphotericin B MICs. Broad MIC ranges are obtained by Etest (34, 106, 152, 154, 221), and the three groups who reported conflicting data with the broth-based system have reported quite comparable agar-based testing results. By the Etest method, cutoff values for resistance of  $\geq 0.5$   $\mu\text{g/ml}$  on Antibiotic Medium 3 agar (106),  $>1$   $\mu\text{g/ml}$  on Antibiotic Medium 3 agar (221), and  $\geq 0.38$   $\mu\text{g/ml}$  on RPMI 1640 agar (34, 152) are suggested. The study with the higher breakpoint was from one of our laboratories (J.H.R.) and used

an older lot of Antibiotic Medium 3 agar—most recent work with newer lots of AM3 found that lower breakpoints are more suitable for these newer lots (unpublished data).

In practice, none of these cutoff values should be treated as a confirmed interpretive breakpoint without significant additional testing. Use of adequate control isolates is critical for testing with any format, and well characterized amphotericin B-resistant *Candida* isolates have been deposited with the American Type Culture Collection (ATCC 200950, ATCC 200951, ATCC 200952, ATCC 200953, ATCC 200955, and ATCC 200956 are resistant, and ATCC 200954 is susceptible).

**(i) Pharmacodynamic support for these ideas.** Available in vitro data demonstrate that the effect of amphotericin B is concentration dependent (23, 99). This pharmacodynamic pattern has also been shown for nystatin, a related polyene antifungal (85). The in vivo prediction of such a result is that the most relevant pharmacodynamic correlate should be the ratio of peak drug concentration to MIC, and precisely this in vivo result was recently shown for amphotericin B in a murine model of invasive candidiasis (5). These data can be combined in an effort to predict the likely clinical response for isolates with differing amphotericin B MICs. In the in vitro studies with this drug, peak/MIC ratios of  $\sim 2$  correlated with near-maximal activity. The pharmacology of amphotericin B is poorly understood, but peak levels of 1 to 2  $\mu\text{g/ml}$  are typical (19, 82; I. Bekersky, R. M. Fielding, D. Dressler, D. N. Buell, and T. J. Walsh, Program Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 85S, 2000; W. G. Powderly, G. G. Granich, G. P. Herzig, and D. J. Krogstad, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 233, 1987). Taking the lower end of the range of levels in blood as a conservative estimate of the achieved peak level, amphotericin B MICs of  $\sim 0.5$   $\mu\text{g/ml}$  might thus represent the upper end of the treatable range, and this is consistent with estimates reported above. However, this estimate is made problematic by technical factors that influence amphotericin B MIC determinations, as discussed above. It is further unclear how to interpret the blood levels obtained with lipid-associated formulations of amphotericin B (82, 187).

**Flucytosine, *Candida* spp., and *C. neoformans*.** When flucytosine is used as monotherapy for yeast infections, there is a high tendency for rapid development of resistance (70, 89). This agent has therefore been used almost always in combination with other antifungal agents, and there are no (and never will be any) extensive data on correlations of MIC with outcome of flucytosine monotherapy. Thus, the M27-A document (135) proposed interpretive breakpoints based on a combination of historical data and results from animal studies (49, 206). Isolates with a flucytosine MIC of  $\leq 4$   $\mu\text{g/ml}$  are said to be susceptible, those with a flucytosine MIC of  $>16$   $\mu\text{g/ml}$  are said to be resistant, and those in between are said to be intermediate. MIC distributions for collections of yeasts are strongly bimodal, with relatively few isolates having an intermediate flucytosine MIC. These breakpoints are described primarily for *Candida* spp. but may well also apply to *C. neoformans*. As an example of this, Hospenthal and Bennett provided a recent review of their experience with flucytosine monotherapy for cryptococcal meningitis (89). In the patients in whom resistance developed, MICs rose from  $<5$  to  $>320$   $\mu\text{g/ml}$ .

(i) **Pharmacodynamic support for these ideas.** In a recent study, flucytosine was found to show concentration-independent activity in time-kill assays, with little additive effect beyond a concentration fourfold higher than the MIC (110). The usual doses of flucytosine (25 mg/kg every 6 h) produce levels of ~40 to 60  $\mu\text{g/ml}$  in blood (70), suggesting an upper end for the treatable MIC range of 10 to 15  $\mu\text{g/ml}$ . In addition to validating the putative interpretive breakpoints, this suggests that lower levels of flucytosine in blood might be just as effective. Such lower levels would also presumably further reduce the rate of toxicity. Andes et al. generated in vivo data supporting this observation by using a murine model of disseminated candidiasis (7). They concluded that a dosing regimen in which the drug maintained levels greater than the MIC for 25 to 50% of the dosing interval would be adequate.

***C. neoformans*, fluconazole, and amphotericin B.** The M27 method includes details for testing *C. neoformans* but does not specify any interpretive breakpoints for agents against this fungus. Although an early report suggested that recurrent cryptococcal meningitis in HIV-infected patients was mostly a function of immune status (28), many subsequent reports have shown the potential for development of true microbiological resistance (1, 10, 39, 167, 185, 227). The basis of resistance is under investigation (150). From a practical standpoint, detection of susceptibility and resistance to both fluconazole and amphotericin B appears to require modification of the reference testing method.

For fluconazole, initial work suggested that testing in yeast nitrogen base (YNB) with a slightly larger than usual inoculum ( $10^4$  CFU/ml) might yield more clinically meaningful MICs (73). This concept has been supported by three clinical data sets (1, 93, 227). In the first study (227), MICs for isolates from 86 HIV-infected and fluconazole-treated patients with cryptococcal meningitis were measured. Testing by the reference method (RPMI 1640 medium) showed little correlation with outcome. On the other hand, MICs obtained by the modified YNB-based method showed a good correlation with outcome. In the second study, this modified method was used to test isolates from a group of 120 HIV-infected and fluconazole-treated patients with cryptococcal meningitis. Although clinical data were limited to 35 of these patients, the YNB-based method again demonstrated an overall correlation with outcome. MICs obtained by the M27 reference method were, however, tightly clustered in a very narrow range and did not show any trend toward correlation with outcome. Unfortunately, neither of these studies provides adequate data for an estimation of interpretive breakpoints, but both suggest that failure rates begin to rise noticeably once the MIC determined by YNB-based method reaches 2 to 4  $\mu\text{g/ml}$ . In the third study, the YNB-based modification of M27-A was employed exclusively, and clinical failure was uniformly seen when the MIC was  $\geq 16$   $\mu\text{g/ml}$  (1).

As with *Candida* spp., consistent detection of amphotericin B resistance in *C. neoformans* has proven difficult. Again, testing on agar or use of Antibiotic Medium 3 broth appears to be helpful. Using a highly selected set of isolates from patients with cryptococcal meningitis and known responses to amphotericin B, Lozano-Chiu et al. demonstrated that the reference method did not reliably distinguish putatively amphotericin B-susceptible and -resistant isolates (117). As was the case with

*Candida* spp., separation could be discerned by repeated testing, but results from any individual test run were too narrowly clustered to permit reliable differentiation. Use of Antibiotic Medium 3 broth provided good separation between resistant and susceptible isolates, but the best results were obtained with Etest on either RPMI 1640 or Antibiotic Medium 3 agar. Isolates from five patients with cryptococcal meningitis who failed to respond to initial therapy with amphotericin B were shown to be tolerant (i.e., loss of the usual fungicidal effect) to amphotericin B in a study by Rodero et al. (185). The same group subsequently reported that MFCs might yield the best correlation with therapeutic response (186); however, correlation was incomplete, and further work is clearly required.

Pharmacodynamic studies with *C. neoformans* have only recently begun to be reported (22, 53, 100, 110), and the behavior of the azoles and polyenes for *C. neoformans* appears similar to that for *Candida* spp. In vivo studies have not yet been reported.

### Molds

Interpretation of mould MICs has long been known to be problematic. For example, Dixon and Polak found no relationship between agar dilution MICs of amphotericin B, flucytosine, ketoconazole, fluconazole, amorolfine, and terbinafine and therapeutic outcome in experimental infection with *Cladophialophora bantiana*, *Ochroconis constricta*, and *Wangiella (Exophiala) dermatitidis* (49). Likewise, an extensive study of nine mold isolates (two *Aspergillus* spp., three *Fusarium* spp., two *Pseudallescheria boydii* strains, and two *Rhizopus arrhizus* strains) found that amphotericin B and itraconazole MICs obtained by the NCCLS reference method provided few clues to the likelihood of in vivo response (144). Uncontrolled host response variables presumably confounded any potential in vivo-in vitro correlations. This exhaustive study also illustrated the limitations of in vivo models—interpretable in vivo systems could not be established for all organism-drug combinations.

Using a collection of carefully selected isolates of *A. fumigatus*, Denning and coworkers have made substantial progress in this area (45, 46, 96) and have recently reviewed the available information on antifungal drug resistance in *Aspergillus* (130). The most definitive work is in the area of detection of azole resistance. Two isolates (NPCF 7099 and NPCF 7100) were collected from patients who did not respond to therapy with itraconazole. These isolates were resistant to itraconazole in a murine model of invasive aspergillosis and had elevated itraconazole MICs (45, 46). The choice of assay system was critical in the detection of these elevated MICs. For microdilution testing, the recommended conditions were 0.165 M MOPS-buffered RPMI 1640 medium (pH 7.0) supplemented to 20 g/liter with glucose, an inoculum of  $10^6$  conidia/ml, incubation at 35°C, and MIC determination after 48 h as the lowest drug concentration that produced no more than trace growth (45). For agar dilution-based testing, the optimum system consisted of similarly buffered but glucose-supplemented RPMI 1640, an inoculum of  $10^6$  to  $10^7$  conidia/ml, incubation at 35°C, and end-point determination after 48 to 72 h as the lowest concentration yielding no growth. These conditions gave the best correlation with in vivo treatment outcome in the animal model.

Unfortunately, the observations just discussed did not, in these investigators' hands, extend to amphotericin B. An isolate of *A. fumigatus* (AF65; deposited in the National Collection of Pathogenic Fungi, Bristol, United Kingdom, as NCPF 7097) from a patient with pulmonary aspergillosis who responded incompletely to high-dose amphotericin B and an isolate of the intrinsically amphotericin B-resistant species *A. terreus* (Walsh et al., 14th Congr. ISHAM) were both unresponsive to amphotericin B in murine models of invasive aspergillosis (96, 215). Only the *A. terreus* isolate tested resistant in vitro. A wide range of media and test conditions (including Antibiotic Medium 3) failed to distinguish the resistant *A. fumigatus* isolate from a pair of isolates that were proven susceptible in vivo.

Since amphotericin B-resistant mutants of *A. fumigatus* can be generated and detected in vitro (119, 121) and the isolate of *A. terreus* did test resistant, the above difficulty in identifying the resistant isolate of *A. fumigatus* is surprising. Using a straightforward adaptation of the NCCLS M27 methodology, Lass-Flörl et al. demonstrated a good correlation between amphotericin B MIC, of  $\geq 2 \mu\text{g/ml}$  and an increased likelihood of clinical failure in patients with hematological malignancies (104).

Considered as a whole, these studies with filamentous fungi provide a cautionary note regarding the interpretation of mold MICs. Underlying diseases and technical laboratory factors are always important, but they may be especially so for invasive mold infections. It would appear that testing of *A. fumigatus* isolates against azole antifungal agents has significant potential value, and the interested reader is referred to the detailed review by Moore et al. on this topic (130). Testing of amphotericin B is, as for the yeasts, problematic. The most useful information is often derived from complete identification of the fungus itself. As examples of the value of identification to the species level, isolates of *Aspergillus terreus* (104, 207; Walsh et al., 14th Congr. ISHAM), *Scedosporium apiospermum* (*Pseudallescheria boydii*) (57, 219), and *Scedosporium prolificans* (17) are usually resistant to amphotericin B.

### Other Drugs

Using the M27-A methodology, Pelletier et al. reported emergence of resistance to clotrimazole in a prospectively monitored HIV-infected pediatric population (149). If the clotrimazole MIC for the infecting *C. albicans* isolate was  $\geq 0.5 \mu\text{g/ml}$ , there was a significant risk ( $P < 0.001$ ) of cross-resistance to other azoles and a risk of clinically overt failure of antifungal azole therapy.

The allylamine terbinafine is widely used for treatment of nail, skin, and hair infections caused by both dermatophyte and nondermatophyte fungi (44, 109, 212). Susceptibility testing of molds and yeasts against terbinafine has been reported with NCCLS M-27A, M-38P, and modifications of these methods (94, 101, 124, 126, 140, 192). The relevance of terbinafine susceptibility testing, however, remains uncertain. For example, one study found that the identity of the etiologic fungus and not the in vitro MIC was more likely to predict the outcome of therapy for tinea capitis (129).

A number of other antifungal agents are now entering the clinical arena. These include new azoles (ravuconazole, vori-

conazole, and posaconazole) and agents of the glucan synthesis inhibitor groups (caspofungin, FK463, and anidulafungin). QC data for yeasts with the M-27 method are now available for voriconazole, ravuconazole, posaconazole, caspofungin, and anidulafungin (14). Clinically relevant methods and interpretive breakpoints for these agents are unknown. The azole antifungal agents will presumably provide reasonable correlations with an M27-A like method. Whether M27-A will be a suitable method for the glucan synthesis inhibitors is not currently known, and available data suggest that new approaches be required for in vitro prediction of their in vivo activity (103; C. M. Douglas, J. C. Bowman, G. K. Abruzzo, A. M. Flattery, C. J. Gill, L. Kong, C. Leighton, J. G. Smith, V. B. Pikounis, K. Bartizal, M. B. Kurtz, and H. Rosen, Program Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1683, 2000).

### CONCLUSIONS

Antifungal susceptibility testing has evolved rapidly during the last decade and has now become a relevant clinical tool (Table 2). However, susceptibility testing is not an infallible answer to questions about treatment of fungal diseases. Routine susceptibility testing is appropriate for *Candida* isolates (especially non-*albicans* species) from deep sites (160) but not at present for other fungi or settings. Testing of *Candida* isolates from the oropharynges of patients who fail to respond to standard therapy can help distinguish microbiological failures from other causes (e.g., noncompliance or drug interactions). Importantly, knowledge of the species of *Candida* is a useful guide to the probable pattern of susceptibility, and identification can be obtained in less than 1 day with some of the newer identification systems. Testing of isolates of *C. neoformans* might be pursued cautiously, since there are strong suggestions of relevant correlations. Mold fungi should not at present be routinely tested for susceptibility. They should, however, be identified to the species level since this often gives very useful therapeutic clues.

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