SPECIAL ARTICLE

The Evolving Role of Antifungal Susceptibility Testing

Gregory A. Eschenauer and Peggy L. Carver

Although increasing numbers of hospital microbiology laboratories are performing antifungal susceptibility testing (AST), its routine use is uncommon. The utility of AST is founded on the belief that susceptibility (or resistance) of an agent allows some prediction of clinical outcome. This review provides an overview of the development of antifungal susceptibility testing methodology, including wild-type minimum inhibitory concentration (MIC) distributions, epidemiologic breakpoints, and Interpretive Clinical Breakpoints for antifungal agents. In addition, we examine the current clinical utility of AST and the clinical data support utilized in the development of clinical breakpoints (CBP) for common pathogens causing invasive fungal infections. In the treatment of fungal infections, identifying consistent correlations between MICs – or susceptibility category – and clinical outcomes is an ongoing challenge, and current data sets are insufficient for many drugs and pathogens to enable the development, revision, or confirmation of CBPs. Antifungal susceptibility testing is of current value, but further research in many areas is needed before MICs are independently used to guide treatment decisions.

Key Words: antifungals, antifungal susceptibility testing, clinical breakpoints, resistance, echinocandin, azole antifungal, fluconazole, epidemiological cut-off value.

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An increasing number of hospital microbiology laboratories are performing antifungal susceptibility testing (AST). Routine use of AST, however, is uncommon. In 2003, a questionnaire was sent to the microbiology departments of 386 randomly selected teaching hospitals in the United States inquiring about the use of AST. Of the 171 respondents, AST was reported by 115 (67.2%) of hospitals (predominantly for *Candida* blood stream infections); however, only 27 hospitals reported on-site testing.[1] Several experts and consensus groups have recommended routine fluconazole susceptibility testing of *Candida* species isolated from sterile sites.[2] Increased interest in AST has been stimulated by an increased number of available antifungal agents, the changing epidemiology of fungal infections (with a greater proportion of non-*albicans* species causing invasive candidiasis), the increased incidence of resistant pathogens, and the increased availability of commercial susceptibility testing systems.[3, 4] Arguably, one of the primary roles of susceptibility testing is to detect resistance, and thereby determine which agents will not be efficacious; thus, the utility of AST is founded on the belief that susceptibility (or resistance) of an agent allows some prediction of clinical outcome. Therefore, we chose to

From the University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania (G.A. Eschenauer) and the University of Michigan College of Pharmacy, Ann Arbor, Michigan (P. L. Carver).

For questions or comments, contact Peggy L. Carver, Associate Professor of Pharmacy, Department of Clinical, Social, and Administrative Sciences, University of Michigan College of Pharmacy, 428 Church Street, Ann Arbor, MI 48109-1065; e-mail: peg@umich.edu.

examine the current clinical utility of AST and the development of clinical breakpoints (CBPs) for two common pathogens–*Candida* species and *Aspergillus*–causing invasive fungal infections.

Overview of the Development of Antifungal Susceptibility Testing Methodology and Interpretive Clinical Breakpoints for Antifungal Agents

Since the late 1980s, the Clinical and Laboratory Standards Institute (CLSI), known until 2005 as the National Committee on Clinical Laboratory Standards (NCCLS), has worked toward the development of standardized in vitro susceptibility testing methods for fungi, with the ultimate goal of establishing CBPs for fungal pathogens. Early efforts focused on the development of guidelines for in vitro susceptibility testing methods. These were published in 1997 as document M27-A for testing fluconazole susceptibility against *Candida* species, and later, in documents M38-A2 and M51-A for filamentous fungi. Although an in-depth discussion of in vitro methodology is beyond the scope of this review, the goal of CLSI has been to develop rapid, reproducible methods that produce concordant results with a variety of techniques such as broth macro- or microdilution, E-test, and automated methodologies.[2, 5, 6]

In the United States, CLSI defined CBPs for fluconazole, itraconazole, voriconazole, and flucytosine for all *Candida* species.[2] No CBPs have been established for posaconazole or amphotericin B versus *Candida*. Outside of the United States, a variety of national breakpoint committees, or regulatory authorities, have also defined minimum inhibitory concentration (MIC) breakpoints and methodologies with which to test *Candida* isolates. However, given that antifungal therapy is similar in most countries, it is illogical to consider the same pathogen "susceptible" in one country and "resistant" in another. Further, this makes it difficult to compare resistance rates among countries and to monitor the development of resistance or of strategies designed to ameliorate it; thus, in 2003, there was a call for harmonization of clinical breakpoints between European countries.[7] Recently, CLSI proposed revised species-specific CBPs for fluconazole and voriconazole to reflect new clinical data and to harmonize with the CBPs defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[8, 9]

Clinical Breakpoints versus Epidemiologic Cut-Off Values

Although CBPs are based primarily on pharmacokinetic-pharmacodynamic relationships, they do take into account other factors, such as differences in dosing regimens, toxicology, resistance mechanisms, intended or approved indications for use, clinical outcome data, and wild-type MIC distributions.[7, 10] Clinical breakpoints can be used to differentiate strains for which there is a high likelihood of treatment success (organisms that are clinically susceptible) from those for which treatment is more likely to fail (clinically resistant). A clinically intermediate or susceptible dose-dependent category can be assigned to pathogens for which the level of antimicrobial agent activity is associated with uncertain therapeutic effect, implying that infections due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used.[7]

Although CBPs are designed to guide therapy, they do not distinguish between isolates with or without resistance mechanisms, nor do they always allow for their early detection. Rather, a "normal range" or "wild-type distribution" of MIC values can be compiled for each pathogen. A wild-type strain is defined by EUCAST as one in which there is an absence of acquired and mutational resistance mechanisms to the antimicrobial agent in question.[7, 11] In general, the MIC distribution for a wild-type organism covers three to five 2-fold dilutions around the modal MIC.[10, 12] When comparable methodologies are utilized, wild-type MIC distributions for a given pathogen are similar worldwide.[10] One advantage of defining normal ranges of MIC values is that nonwild-type organisms with acquired resistance mechanisms can be identified. These nonwild-type organisms have higher MICs compared with the upper limit of the wild-type distribution, which is defined as the epidemiologic cut-off value (ECV) MIC. The MIC value of the ECV generally encompasses at least 95% of the isolates in the wild-type distribution.[11, 13, 14] Figure 1 illustrates a typical wild-type distribution, ECV, and CBPs for an "example" antifungal. Note that the few isolates with high level ($\geq$512 ug/ml) MICs are above the ECV and have acquired resistance mechanisms. Because it is species specific, the ECV will not be altered by changing circumstances. This is especially

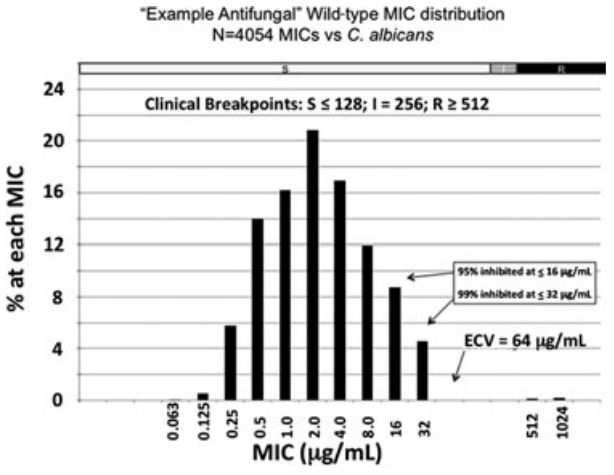

Figure 1. "Example antifungal" wild-type MIC distribution MICs versus *Candida albicans* (n=4054)

useful when there is a gap in MICs between the upper end of the wild-type distribution and the CBP, when the CBP divides two wild-type populations, or when resistance has yet to be described for a pathogen.[7]

The CLSI and EUCAST may refrain from setting breakpoints if the species is considered a poor target for the drug or if there is insufficient evidence that the species is a good target for the drug. As well, CBPs may change over time, as circumstances change.[8] Although organisms with an MIC that exceeds the ECV show reduced susceptibility compared with the wild-type population, they may respond to clinical treatment if their MICs lie below the CBPs. At least one author has suggested limiting the term "resistant" to CBPs and recommends that surveillance reports include summaries of susceptibility data for both CBPs (to describe clinical resistance) and ECVs (to describe pathogens with reduced susceptibility due to the acquisition of resistance mechanisms).[15]

Clinical Data Support for Current Breakpoints

The process for establishing MIC breakpoints involves a compromise among clinical, epidemiologic, and methodologic aspects and ideally provides a correlation between isolates categorized as "susceptible" or "resistant" and clinical outcome. One study has noted that when sufficiently large data sets can be employed to examine the correlation between therapeutic outcome and in vitro susceptibility, dose- (or area under the concentration curve [AUC]) response curves can be generated.[6] Once a response plateau is achieved, further increases in dose (or AUC) produce little effect. Another way of illustrating this plateau effect is the "90-60 rule," which can be applied to determine CBPs. Stated briefly, approximately 90% of infections caused by susceptible isolates respond to appropriate antimicrobial therapy; however, approximately 60% of infections caused by resistant isolates, or for which inappropriate antimicrobial agents are utilized for treatment, also respond. This "90-60 rule" appears to hold true regardless of whether the outcome measurement is clinical response, microbiologic response, or mortality, and whether the in vitro prediction tool is MIC, an inhibition-zone diameter, or the ratio of AUC:MIC. Failure of an individual clinical trial to support the "90-60 rule" is often due to a lack of sufficient drug-resistant isolates in the trial.[6] Unfortunately, the compiled data sets for many MIC-outcome relationships also suffer from small numbers of comparisons (especially in regard to drug-resistant isolates). It is important to note that some infections will not respond by increasing the dose, whereas others do so despite the use of "small" doses—or even without therapy. In addition, factors such as variable pharmacokinetics, drug delivery to the site of infection, host defenses, and toxin production all contribute to patient response.

Candida species

Fluconazole

Fluconazole exhibits time-dependent fungistatic activity against *Candida* species, and in vitro and in vivo models demonstrate correlations between fluconazole dosage, the pathogen fluconazole MIC, and outcomes. In a neutropenic *Candida albicans* murine model, the pharmacodynamic parameter associated with efficacy was an AUC:MIC of approximately 25.[16] After administration of fluconazole doses of 400–2000 mg/day to healthy adults with normal renal function, AUC was virtually equivalent to the daily dosage.[17, 18] Thus, fluconazole doses of 400 or 800 mg/day, respectively, result in an AUC:MIC of 25 for isolates with MICs of 16 or 32.

Interpretive breakpoints for MIC testing of fluconazole against *Candida* species were initially proposed in 1997 by the NCCLS (now CLSI) Subcommittee for Antifungal Testing, and later published as document M27-A. They were based on the accumulated clinical outcome and microbiologic data available at that time.[2] However,

there are now concerns regarding the relative paucity of data to support these breakpoints. First, almost 80% of the outcome data were obtained from patients with mucosal rather than invasive disease; although there appears to be a clear relationship between susceptibility category and outcome in mucosal infections, the relationship for invasive infections is less evident. Second, few clinical outcome data were available in isolates with elevated ($\geq$16 μg/ml) MICs.[2]

Given the limitations of the original data set, a significant amount of subsequent research has been devoted to examining the recently revised breakpoint recommendations, which consisted largely of retrospective studies that attempted to correlate dose:MIC ratios with efficacy.[2] One early study categorized outcomes by the (then) new breakpoints and found that the rates of clinical cure in patients with invasive candidiasis treated with 400mg/day fluconazole were 79% (19 of 24), 66% (four of six), and 0% (0 of 2) for infections caused by susceptible, susceptible dose-dependent, and resistant isolates, respectively.[19] We reviewed the dose:MIC studies,[18, 20–23] which explicitly segregated data for patients with invasive infections and for which correlations of dose:MIC with outcome were analyzed (Table 1). Trials that did not include explicit dose information or did not analyze fluconazole outcomes separately from other agents were not included.[5, 24] Some studies provided fluconazole dosages without evaluating the impact of renal function on the effective dosage administered, making the assessment of dose:MIC or AUC:MIC difficult to assess. In addition, the definition of therapeutic failure differed between studies and two solely assessed mortality, which may not be the most appropriate determinant of treatment success.[22, 23] Taken together, the available data do not present a consistent dose:MIC relationship and further illustrate the difficulty in obtaining a robust data set of isolates with elevated MICs.

A 2010 article proposed significantly lower breakpoint MICs for non-*glabrata* species for four of the most commonly encountered *Candida* species, based on distribution data demonstrating a very low prevalence of MICs above these breakpoints.[8] The "susceptible" category was deleted for *C. glabrata*, and all isolates with MICs of 32 μg/ml or less were termed "susceptible dose-dependent" (Table 2).[8, 9, 25, 26] However, the supporting data are again limited; there appears to be no relationship between *C. tropicalis* MIC and outcome, clinical data in support of the *C. parapsilosis* breakpoints are not

Table 1. Studies Evaluating Correlation of Fluconazole Dose:MIC with Outcome in Invasive Candidiasis

	Rex[20]	Clancy[21]	Pai[22]	Rodriguez-Tudela[18]	Baddley 2008[23]
N	113	32	77	126	84
Dose:MIC ratio that correlated with successful outcome	None determined, since an inverse correlation (p=0.05) was found between MIC and outcome. All patients were administered dosages equivalent to 400 mg daily	Dose:MIC > 50 associated with 74% success vs 8% for dose: MIC $\leq$ 50 (p=0.0003).	Dose: MIC = 13.3 $\pm$ 10.5 (mean $\pm$ SD) in survivors vs 7.0 $\pm$ 8.0 in nonsurvivors (p=0.03).	Patients whose dose: MIC $\geq$ 100 were associated with 92% (109/118) success vs 50% (4/8) for those with dose: MIC $\leq$ 100 (no statistics performed)	Dose:MIC = 11.5 (identified by CART analysis, $p \leq 0.09$)
Definition of failure	Persistence of candidemia on therapy	Persistence of candidemia despite 3 days of therapy, or breakthrough candidemia while receiving fluconazole for $\geq$ 3 days as empiric therapy	Mortality	Persistence of candidemia despite at least 4 days of fluconazole therapy	Mortality
No. of isolates with elevated MICs	6 with MICs $\geq$ 16	8 with MICs $\geq$ 16	4 with MICs $\geq$ 16	4 with MICs $\geq$ 16	9 with MICs $\geq$ 16

MIC = minimum inhibitory concentration, CART = Correlation and Regression Trees.

Table 2. Interpretive Breakpoints

Current Interpretive Clinical Breakpoints (CLSI M27-S3)[25]			
	Susceptible	Susceptible-Dose Dependent	Resistant
Fluconazole	≤ 8	16–32	≥ 64
Voriconazole	≤ 1	2	≥ 4
Itraconazole	≤ 0.125	0.25–0.5	≥ 1
	Susceptible	Intermediate	Resistant
Flucytosine	≤ 4	8–16	≥ 32
Echinocandins	≤ 2	–	–

provided, and the *C. glabrata* data set remains extremely small (74 cases total, of which only 22 have an MIC of 16 μg/ml or more).[8]

At this time, the relationship between fluconazole breakpoints and clinical outcome has not been validated by a robust data set that includes a large number of non-*albicans* isolates as well as isolates with elevated MICs. Current (2009) guidelines from the Infectious Diseases Society of America for the management of candidiasis endorse routine susceptibility testing for *C. glabrata* isolates obtained from sterile sites, and deescalation from echinocandin to fluconazole only if susceptibility is documented. Testing is recommended for infections due to other *Candida* species if the patient is not responding to therapy or if resistance to fluconazole is suspected. These recommendations are given evidence grading consistent with expert opinion (B-III).[27]

Itraconazole, Ketoconazole, and Flucytosine

Initial CBPs for itraconazole were derived utilizing outcome data from mucosal disease only, whereas CBPs for ketoconazole have not been proposed.[6] However, wild-type MIC distributions and ECVs for itraconazole and flucytosine were recently reported for seven species (*albicans, tropicalis, parapsilosis, dubliniensis, krusei, guilliermondii, lusitaniae*) of *Candida*. In the absence of species-specific CBPs for these older and less widely utilized antifungal agents, wild-type MIC distributions and ECVs can be used to monitor for the emergence of resistance.[14]

Voriconazole

Revised CBPs (Table 3) were recently proposed for the five most commonly encountered *Candida* species and voriconazole.[9] A Correlation and Regression Trees analysis of 47 isolates of *C. glabrata* was unable to discern an interpretive breakpoint differentiating success and failures for voriconazole with *C. glabrata*, and only nine *C. krusei* isolates were available for analysis. An MIC of 0.125 μg/ml optimally discriminated between success and failure for *C. albicans*, *C. tropicalis*, and *C. parapsilosis*.[9, 28] With the exception of *C. krusei*, most *Candida*

Table 3. Proposed Interpretive Clinical Breakpoints[8, 9, 26]

	Susceptible	Susceptible-Dose Dependent	Resistant
Fluconazole		*C. albicans*, *C. tropicalis*, and *C. parapsilosis*	
	≤ 2	4	≥ 8
		C. glabrata	
	—	≤ 32	≥ 64
	Susceptible	Intermediate	Resistant
Voriconazole		*C. albicans*, *C. tropicalis* and *parapsilosis*	
	≤ 0.125	0.25–0.5	≥ 1
		C. krusei	
	≤ 0.5	1	≥ 2
Caspofungin		*C. albicans*, *C. tropicalis* and *C. krusei*	
Micafungin	≤ 0.25	0.5	≥ 1
Anidulafungin		*C. parapsilosis*	
	≤ 2	4	≥ 8
Caspofungin		*C. glabrata*	
Anidulafungin	≤ 0.12	0.25	≥ 0.5
Micafungin	≤ 0.06	0.12	≥ 0.25
Posaconazole		Interpretive criteria have not been established	
Amphotericin B		Interpretive criteria have not been established	

CLSI = Clinical and Laboratory Standards Institute.

species exhibit cross-resistance across the azole class, and voriconazole does not display substantial activity against fluconazole-resistant *C. glabrata*.[29] As such, voriconazole has a limited role in the treatment of candidiasis.

There has been significant interest in therapeutic drug monitoring of voriconazole, due to its unpredictable patient-to-patient pharmacokinetics. A recent study correlated voriconazole trough levels (extrapolated from a PK model) with clinical outcomes, utilizing data from several trials of invasive candidiasis and aspergillosis. Monte Carlo simulation revealed a relationship between the calculated trough:MIC ratio, with near-maximal attainment of successful outcomes with trough:MIC values of 2–5. Limitations to this trial include the use of modeled trough levels, the fact that separate results are not presented for molds and yeasts, and that patients who discontinued voriconazole (who constituted a significant proportion of failures in some trials) were included in the analysis as therapeutic failures.[30]

Amphotericin B

Clinical breakpoints have not been established for amphotericin B and *Candida* species, as there are conflicting data regarding the correlation between amphotericin B MICs and invasive candidiasis outcomes. An early study of 26 patients with hematologic malignancies who developed candidemia found a significantly higher mortality rate in patients infected by isolates with MICs greater than 0.8 μg/ml compared with MICs of 0.8 μg/ml or less (100% vs 47%, p=0.04). However, it is possible that the results were influenced by differences in baseline characteristics between the groups. For example, more than twice as many patients who died received allogeneic transplants compared with those who survived. In addition, the wide range of MICs reported in this study (0.4– 7.5 μg/ml) was unusual.[31] In a randomized trial comparing amphotericin B with fluconazole for the treatment of invasive candidiasis, the distribution of amphotericin B MICs was too narrow to define a relationship to outcome.[20]

Subsequent studies have examined whether different techniques for determining MICs might elucidate a clinically meaningful MIC breakpoint. One study of 105 patients with candidemia found no significant correlation between MICs (determined by broth macrodilution) and microbiologic failure, but did find that all five patients with 48-hour MICs of 1 or greater failed therapy (vs 28/100 with 48-hour MICs < 1).[32] The authors subsequently explored whether determination of MIC by E-test might better discriminate between successes and failures. Measured at 48 hours, an MIC of 0.38 μg/ml or more was predictive of failure (56% failure vs 16% for isolates with MICs < 0.38 μg/ml, p=0.0001).[33] Although significant results were detailed, this trial illustrated the narrow range of MICs described above, and suggests that reproducibility of such small variations with the E-test methodology may be challenging. A subsequent study that attempted to correlate a specific test method (broth microdilution with two different media or E-test) with therapeutic failure (defined as death or persistent or recurrent fever while on therapy) was unable to confirm a significant relationship.[34]

In conclusion, insufficient evidence exists to support a clinically relevant MIC breakpoint for amphotericin B to *Candida*. Negative outcomes may be more common for infections due to isolates with MICs of 1 μg/ml or more, but these data still require validation; however, wild-type MIC distributions and ECVs for amphotericin B were recently reported for seven species (*albicans, tropicalis, parapsilosis, dubliniensis, krusei, guilliermondii, lusitaniae*) of *Candida*. In the absence of species-specific CBPs, wild-type MIC distributions and ECVs can be used to monitor for the emergence of resistance.[14]

Echinocandins

The correlation of outcome with echinocandin MIC is currently a matter of intense scrutiny and debate. Revised CBPs (Table 3) were recently proposed for the five most commonly encountered *Candida* species and the three available echinocandins.[26] The impetus for this revision was the result of emerging data suggesting that previously proposed CBPs for the echinocandins (with "susceptible" defined as ≤ 2 μg/ml) failed to segregate isolates with *FKS* "hot spot" mutations.[35] Echinocandins inhibit glucan synthase, an enzyme that consists of at least two subunits – Fksp and Rho1p. The Fksp subunit of glucan synthase, which is encoded by *fks1, fks2*, and *fks3*, catalyzes the biosynthesis of (1,3)-β-D-glucan, an essential component of the cell wall of many fungi, including *Candida*. MICs for isolates with Fks1p or Fks2p mutations, while elevated above wild-type MICs, may be as low as 0.12 μg/ml (for caspofungin).[26] Recent

reports have proposed a correlation between *FKS* "hot spot" mutations and decreased response rates in animal models as well as in clinical failures of echinocandin therapy. Murine models have confirmed that echinocandin therapy (even at elevated doses) is not consistently effective against infections due to isolates with *fks1* or *fks2* "hot spot" mutations.[36–39] Given these data, the CBPs have been substantially lowered to delineate between true wild-type isolates and those isolates that potentially harbor mutations (Table 2).[26]

However, there are several concerns regarding the CLSI approach to the echinocandin breakpoint revisions. First, some institutions report generally higher caspofungin MICs than those reported in the CLSI CBP revision rationale document. In a study from the University of Pittsburgh, all 39 isolates of *C. glabrata* tested (using CLSI methodology) displayed caspofungin MICs above the revised CBP (100% resistance). Despite this, 74% of patients responded to echinocandin therapy (with 36/39 patients treated with caspofungin).[40] Second, it may be that isolates that do not harbor *FKS* mutations but have MICs above the revised CBPs will now be identified as "resistant" when echinocandin therapy may still be successful. In fact, multivariate analysis revealed *FKS* mutation, not MIC, to be the only independent risk factor associated with echinocandin failure in infections due to *C. glabrata*.[40]

The available clinical trial data also fail to support the revised CBPs. In an early study of the treatment of invasive candidiasis, treatment response was evaluated by caspofungin MIC. The MIC_{90} for all 231 isolates was 2 μg/ml (96% with MICs 0.125–2 μg/ml). The authors found no correlation between MIC and outcome.[41] In another randomized, double-blind trial comparing amphotericin B with caspofungin in patients with invasive candidiasis, all infections due to isolates with caspofungin MICs greater than 2 μg/ml responded to therapy.[42]

In conclusion, there are insufficient data from which to base decisions regarding echinocandin therapy based strictly on elevated MICs, especially regarding *C. glabrata*.

Cryptococcus Species

Although MIC and zone diameter interpretive breakpoints have not been established for any antifungal against *C. neoformans*, one recent study reported the establishment of wild-type MIC distributions and ECVs for fluconazole, posaconazole, and voriconazole when testing *Cryptococcus neoformans* by microdilution methods. This information will be useful in detecting the emergence of isolates with reduced azole susceptibility (nonwild-type strains) in *C. neoformans*. Application of the ECV to MIC testing by the CLSI microdilution method to 986 global clinical isolates obtained over a 13-year period, allowed evaluation of trends in fluconazole susceptibility. The modal MIC of fluconazole remained unchanged over the time period; however, the proportion of nonwild-type isolates that exceeded the ECV decreased progressively from 4.2% in 1996–2000 to only 0.5% in 2005–2008.[43]

Similarly, another author reported wild-type MICs and preliminary ECVs for four triazole antifungals (fluconazole, voriconazole, itraconazole, and posaconazole) in *C. gattii*.[44] Although *C. gattii* has long been known to cause cryptococcal infection in tropical and subtropic areas of the world, it has recently emerged as a pathogen that can cause severe disease in otherwise healthy individuals in the U.S. Pacific Northwest and in British Columbia. Although isolates of *C. gattii* are less susceptible to triazoles than are isolates of *C. neoformans*, it is unclear whether different drug therapy is needed for infections caused by the two species. As *C. gattii* appears to consist of four molecular types, each of which has different geometric mean MICs to triazoles, preliminary ECVs differ for each triazole and molecular type.[44] Most recently, ECVs have been established for amphotericin B and flucytosine in *C. neoformans* and *C. gattii*.[45]

Fluconazole MICs are predictive of treatment failure in animal models and in clinical cases of relapse.[43] Limited data suggest that patients infected with isolates with fluconazole MICs of 8 or less respond better than those with isolates having MICs of 16 μg/ml or more.[46] In a small, retrospective study of 27 patients infected with HIV and cryptococcal meningoencephalitis, 76% of culture-positive relapses were associated with isolates having MICs of 16 μg/ml or more.[47] Although *C. neoformans* appears more susceptible in vitro to posaconazole and voriconazole than fluconazole,[43] few outcome data are available for these agents.[48]

A recent study of 85 patients treated with amphotericin B deoxycholate (0.7 mg/kg/day) for 14 days found a significant association between MIC (determined by CLSI method) and survival at day 14 ($p=0.03$) but not at day 28

(p=0.09). Survival at day 14 was 97% (35/36) for MICs of 0.25 μg/ml or less, 87% (26/30) for MIC 0.5 μg/ml, and 67% (12/18) for MICs of 1 μg/ml or more. However, MIC did not correlate with quantitative mycologic response in the cerebrospinal fluid at day 14. In addition, the authors found that an initial in vitro quantitative response of less than 10 colony-forming units/ml to 1.5 μg/ml of amphotericin B (using an inoculum that matched the number of organisms present in the baseline cerebrospinal fluid) was associated with survival. They suggest that since no clear correlation has been found between MICs and outcome for amphotericin B and cryptococcal infections (perhaps again due to the narrow range of MICs obtained), such novel "susceptibility testing" techniques are perhaps necessary to derive meaningful information.[49]

Current guidelines for the management of cryptococcal infections recommend susceptibility testing only for patients in whom primary treatment has failed, patients with relapse, and for those with recent exposure to antifungals.[48] These recommendations are based on data showing that primary resistance to first-line agents (such as the data displayed above for fluconazole) is uncommon,[48] the unclear association between MIC and amphotericin B treatment outcome,[49] and that in vitro flucytosine resistance has shown no antagonism or decreased synergy in in vivo models.[48]

Aspergillus and Other Molds

Overall, there are much fewer data assessing the impact of in vitro resistance on clinical outcome for mold infections than for infections caused by *Candida* species. A multitude of factors complicate the derivation of such an association, including the impact of immunosuppression intensity on outcome, frequent use of more than one drug during the course of therapy, unpredictable bioavailability of certain agents (voriconazole and posaconazole), and the relative rarity of infection (especially with non-*Aspergillus* molds). Compounded by the relative rarity of *in vitro* resistance, the above-noted complications make it exceedingly difficult to conduct a well-designed trial.[50] In general, aside from *Aspergillus*, there are virtually no data assessing the impact of in vitro resistance on clinical outcome. However, it should be noted that susceptibility testing is still widely utilized in the treatment of some of these infections, especially with regard to *Fusarium* and *Scedosporium*. Recent reviews of these pathogens offer clinical approaches to treatment, which are largely based on clinical experience and synergy testing.[51, 52] The remainder of this section will focus on *Aspergillus*.

Azoles and Amphotericin B

The frequency of azole resistance in clinical isolates of *Aspergillus* appears to depend on geography and patient population, and the definition of "azole resistance" differs among studies. A reference laboratory in the Netherlands reported resistance (defined as MICs of $\geq$ 2 to voriconazole, > 16 to itraconazole, and $\geq$ 0.5 to posaconazole) in 12 of 13 isolates of *A. fumigatus*.[53] Conversely, isolates of *A. fumigatus* associated with infections in transplant patients were collected by a surveillance network from 23 centers in the United States and reported a resistance rate (defined as MICs $\geq$ 4 to triazoles) of less than 1%. The investigators also reported low levels of resistance to amphotericin B (MIC > 1) aside from *A. terreus*.[54] In another study, a worldwide collection of 1312 isolates of *A. fumigatus* revealed 98.6% susceptibility to voriconazole (defined as an MIC $\leq$ 1).[55] A reference laboratory in the United Kingdom recently reported that 5% of 400 (unique, nonduplicate) *A. fumigatus* clinical isolates were resistant to itraconazole (defined as MIC > 2 mg/L).[50, 56]

Currently, there is no clear correlation between resistance and clinical outcome for invasive aspergillosis and azole antifungals. The previously mentioned analysis of infections in transplant patients in the United States[54] found no correlation between voriconazole MIC and 6- or 12-week mortality. Survival at 6 weeks was associated with increasing MIC to amphotericin B.[54] Although one series reported a high rate of itraconazole clinical failure in patients infected with resistant isolates,[50, 56] another documented two cases of clinical success with voriconazole monotherapy despite in vitro resistance.[53] Although one study suggested a possible relationship between MIC and outcome, the data presented are too limited to draw robust conclusions.[30]

Echinocandins

In an *A. fumigatus* isolate obtained from a patient failing caspofungin therapy, E-test methodology determined the MIC to be greater

than 32. In addition, caspofungin therapy resulted in significantly lower survival in mice infected with the isolate than therapy with posaconazole, to which it was susceptible. The isolate was found to overexpress the *FKS* gene and grow at a more rapid rate compared with a susceptible isolate.[57] Aside from this case, however, no clear clinical correlation has been found between caspofungin MICs and outcomes for infections due to *Aspergillus*. This is confounded by the fact that caspofungin is rarely utilized as monotherapy for the treatment of invasive mold infections. Although ECVs for caspofungin have been determined,[58] very few clinical isolates from a transplant database were found to have minimum effective concentrations at or above these ECVs.[59] As such, truly resistant isolates appear to be very rare, and at this time, no definitive correlation exists between "susceptibility" and clinical outcome.

Other Benefits

In addition to the useful information provided by antifungal susceptibility testing regarding the treatment of *Candida* infections, there are other benefits to testing. For example, routine testing may enable the creation of an institutional or unit-specific fungal antibiogram. This might assist in the development of protocols and guidelines specific to the epidemiology of that institution.[60] In addition, testing may decrease costs by enabling a de-escalation from expensive agents (e.g., echinocandins) to inexpensive agents such as fluconazole.[3, 61]

Conclusion

Identifying consistent correlations between MICs (or susceptibility category) and clinical outcomes is an ongoing challenge in treating fungal infections. Clearly, more robust data sets are needed to enable the development, revision, or confirmation of clinical breakpoints. However, the role of antifungal susceptibility testing is clearly evolving as seen by recent guidelines that encouraged its routine (candidiasis) or targeted (cryptococcosis) use. In addition, several trends suggest an increasingly valuable role for susceptibility testing, including the following: a dose:MIC relationship to fluconazole outcomes in candidiasis (although a specific threshold has not been defined); a correlation of MICs of 1 μg/ml or more and worse outcomes to amphotericin B in candidiasis; and a relationship between high fluconazole MICs and failures or relapses in cryptococcal infections. In addition, susceptibility testing offers some benefits that are not immediately applicable to the care of a specific patient, such as detection of emerging resistance and bolstering the currently limited data sets. Susceptibility testing may also assist in the appropriate stewardship of antifungal agents as well as development of institution-specific guidelines and protocols. Finally, the continuing evolution of commercial testing methods (including automated systems) makes testing more feasible at many centers.[4] As such, antifungal susceptibility testing is of current value, but further research in many areas is needed before treatment decisions can be solely predicated on MICs.

References

1. **Pai MP, Pendland SL.** Antifungal susceptibility testing in teaching hospitals. Ann Pharmacother 2003;37(2):192–6.
2. **Pfaller MA, Diekema DJ, Sheehan DJ.** Interpretive breakpoints for fluconazole and *Candida* revisited: a blueprint for the future of antifungal susceptibility testing. Clin Microbiol Rev 2006;19:435–47.
3. **Collins CD, Eschenauer GA, Salo SL, Newton DW.** To test or not to test: a cost minimization analysis of susceptibility testing for patients with documented *Candida glabrata* fungemia. J Clin Microbiol 2007;45:1884–8.
4. **Lass-Florl C, Perkhofer S, Mayr A.** In vitro susceptibility testing in fungi: a global perspective on a variety of methods. Mycoses 2010;53:1–11.
5. **Rex JH, Pfaller MA, Galgiani JN, et al.** Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. Clin Infect Dis 1997;24:235–47.
6. **Rex JH, Pfaller MA.** Has antifungal susceptibility testing come of age? Clin Infect Dis 2002;35(8):982–9.
7. **Kahlmeter G, Brown DF, Goldstein FW, et al.** European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. J Antimicrob Chemother 2003;52(2):145–8.
8. **Pfaller MA, Andes D, Diekema DJ, Espinel-Ingroff A, Sheehan D.** Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and Candida: time for harmonization of CLSI and EUCAST broth microdilution methods. Drug Resist Updat 2010;13:180–95.
9. **Pfaller MA, Andes D, Arendrup MC, et al.** Clinical breakpoints for voriconazole and *Candida* spp. revisited: review of microbiologic, molecular, pharmacodynamic, and clinical data as they pertain to the development of species-specific interpretive criteria. Diagn Microbiol Infect Dis 2011;70:330–43.
10. **Kahlmeter G, Brown DFJ.** Harmonization of Antimicrobial breakpoints in Europe – Can it be achieved? Clin Microbiol Newsl 2004;26:187–92.
11. **Turnidge J, Paterson DL.** Setting and revising antibacterial susceptibility breakpoints. Clin Microbiol Rev 2007;20(3):391–408.
12. **Arendrup MC, Kahlmeter G, Rodriguez-Tudela JL, Donnelly JP.** Breakpoints for susceptibility testing should not divide wild-type distributions of important target species. Antimicrob Agents Chemother 2009;53(4):1628–9.
13. **Turnidge J, Kahlmeter G, Kronvall G.** Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clin Microbiol Infect 2006;12(5):418–25.
14. **Pfaller MA, Espinel-Ingroff A, Canton E, et al.** Wild-type MIC distributions and epidemiological cutoff values for Amphotericin

B, flucytosine, and itraconazole and *Candida* spp. as determined by CLSI broth microdilution. J Clin Microbiol 2012;50 (6):2040–6.
15. **Simjee S, Silley P, Werling HO, Bywater R.** Potential confusion regarding the term "resistance" in epidemiological surveys. J Antimicrob Chemother 2008;61(1):228–9.
16. **Andes D, van Ogtrop M.** Characterization and quantitation of the pharmacodynamics of fluconazole in a neutropenic murine disseminated candidiasis infection model. Antimicrob Agents Chemother 1999;43:2116–20.
17. **Louie A, Liu QF, Drusano GL, et al.** Pharmacokinetic studies of fluconazole in rabbits characterizing doses which achieve peak levels in serum and area under the concentration-time curve values which mimic those of high-dose fluconazole in humans. Antimicrob Agents Chemother 1998;42(6):1512–4.
18. **Rodriguez-Tudela JL, Almirante B, Rodriguez-Pardo D, et al.** Correlation of the MIC and dose/MIC ratio of fluconazole to the therapeutic response of patients with mucosal candidiasis and candidemia. Antimicrob Agents Chemother 2007;51: 3599–604.
19. **Lee SC, Fung CP, Huang JS, et al.** Clinical correlates of antifungal macrodilution susceptibility test results for non-AIDS patients with severe Candida infections treated with fluconazole. Antimicrob Agents Chemother 2000;44:2715–8.
20. **Rex JH, Pfaller MA, Barry AL, Nelson PW, Webb CD.** Antifungal susceptibility testing of isolates from a randomized, multicenter trial of fluconazole versus Amphotericin B as treatment of nonneutropenic patients with candidemia. Antimicrob Agents Chemother 1995;39:40–4.
21. **Clancy CJ, Yu VL, Morris AJ, Snydman DR, Nguyen MH.** Fluconazole MIC and the fluconazole dose/MIC ratio correlate with therapeutic response among patients with candidemia. Antimicrob Agents Chemother 2005;49:3171–7.
22. **Pai M, Turpin RS, Garey KW.** Association of fluconazole area under the concentration-time curve/MIC and dose/MIC ratios with mortality in nonneutropenic patients with candidemia. Antimicrob Agents Chemother 2007;51:35–9.
23. **Baddley JW, Patel M, Bhavnani SM, Moser SA, Andes DR.** Association of fluconazole pharmacodynamics with mortality in patients with candidemia. Antimicrob Agents Chemother 2008;52:3022–8.
24. **Takakura S, Fujihara N, Saito T, Kudo T, Iinuma Y, Ichiyama S.** Clinical factors associated with fluconazole resistance and short-term survival in patients with *Candida* bloodstream infection. Eur J Clin Microbiol Infect Dis 2004;23:380–8.
25. **Clinical and Laboratory Standards Institute.** Reference method for broth dilution antifungal susceptibility testing of yeasts. Informational supplement M27-A3. Wayne, PA: Clinical Laboratory Standards Institute Clinical Laboratory Standards Institute; 2008.
26. **Pfaller MA, Diekema DJ, Andes D, et al.** Clinical breakpoints for the echinocandins and *Candida* revisited: integration of molecular, clinical, and microbiological data to arrive at species-specific interpretive criteria. Drug Resist Updat 2011;14:164–76.
27. **Pappas PF, Kauffman CA, Andes D, et al.** Clinical practice guidelines for the management of candidiasis: 2009 update by the infectious diseases society of America. Clin Infect Dis 2009;48:503–35.
28. **European Committee on Antimicrobial Susceptibility Testing.** Voriconazole: rationale for the clinical breakpoints, version 2.0, 2010. Available from http://www.eucast.org. Accessed June 14, 2012.
29. **Pfaller MA, Castenheira M, Lockhart SR, Ahlquist AM, Messer SA, Jones RN.** Frequency of decreased susceptibility and resistance to echinocandins among fluconazole-resistant bloodstream isolates of *Candida glabrata*. J Clin Microbiol 2012;50:1199–203.
30. **Troke PF, Hockey HP, Hope WW.** Observational study of the clinical efficacy of voriconazole and its relationship to plasma concentrations in patients. Antimicrob Agents Chemother 2011;55:4782–8.
31. **Powderly WG, Kobayashi GS, Herzig GP, Medoff F.** Amphotericin B-resistant yeast infection in severely immunocompromised patients. Am J Med 1988;84:826–32.
32. **Nguyen MH, Clancy CJ, Yu VL, et al.** Do in vitro susceptibility data predict the microbiologic response to Amphotericin B? Results of a prospective study of patients with *Candida* fungemia. J Infect Dis 1998;177:425–30.
33. **Clancy CJ, Nguyen MH.** Correlation between in vitro susceptibility determined by E test and response to therapy with Amphotericin B: results from a multicenter prospective study of candidemia. Antimicrob Agents Chemother 1999;43:1289–90.
34. **Park BJ, Arthington-Skaggs BA, Hajjeh RA, et al.** Evaluation of Amphotericin B interpretive breakpoints for *Candida* bloodstream isolates by correlation with therapeutic outcome. Antimicrob Agents Chemother 2006;50:1287–92.
35. **Arendrup MC, Pfaller MA; the Danish Fungaemia Study Group.** Caspofungin etest susceptibility testing of *Candida* species: risk of misclassification of susceptible isolates of *C. glabrata* and *C. krusei* when adopting the revised caspofungin CLSI breakpoints. Antimicrob Agents Chemother 2012;56 (7):3965–8.
36. **Spreghini E, Orlando F, Sanguinetti M, et al.** Comparative effects of micafungin, caspofungin, and anidulafungin against a difficult-to-treat fungal opportunistic pathogen, *Candida glabrata*. Antimicrob Agents Chemother 2012;56(3):1215–22.
37. **Arendrup MC, Garcia-Effron G, Buzina W, et al.** Breakthrough *Aspergillus fumigatus* and *Candida albicans* double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. Antimicrob Agents Chemother 2009;53(3):1185–93.
38. **Slater JL, Howard SJ, Sharp A, Goodwin J, Gregson LM, et al.** Disseminated candidiasis caused by *Candida albicans* with amino acid substitutions in Fks1 at position Ser645 cannot be successfully treated with micafungin. Antimicrob Agents Chemother 2011;55:3075–83.
39. **Wiederhold NP, Najvar LK, Bocanegra RA, Kirkpatrick WR, Patterson TF.** Caspofungin dose escalation for invasive candidiasis due to resistant *Candida albicans*. Antimicrob Agents Chemother 2011;55:3254–60.
40. **Shields RK, Nguyen MH, Press EG, et al.** The presence of an FKS mutation rather than MIC Is an independent risk factor for failure of Echinocandin therapy among patients with invasive candidiasis due to *Candida glabrata*. Antimicrob Agents Chemother 2012;56:4862–9.
41. **Kartsonis N, Killar J, Mixson L, et al.** Caspofungin susceptibility testing of isolates from patients with esophageal candidiasis or invasive candidiasis: relationship of MIC to treatment outcome. Antimicrob Agents Chemother 2005;49:3616–23.
42. **Mora-Duarte J, Betts R, Rotstein C, et al.** Comparison of caspofungin and amphotericin B for invasive candidiasis. N Engl J Med 2002;347:2020–9.
43. **Pfaller MA, Castanheira M, Diekema DJ, Messer SA, Jones RN.** Wild-type MIC distributions and epidemiologic cutoff values for fluconazole, posaconazole, and voriconazole when testing *Cryptococcus neoformans* as determined by the CLSI broth microdilution method. Diagn Microbiol Infect Dis 2011;71:252–9.
44. **Lockhart SR, Iqbal N, Bolden CB, et al.** Epidemiologic cutoff values for triazole drugs in *Cryptococcus gattii*: correlation of molecular type and in vitro susceptibility. Diagn Microbiol Infect Dis 2012;73(2):144–8.
45. **Espinel-Ingroff A, Chowdhary A, Cuenca-Estrella M, et al.** Cryptococcus neoformans-*Cryptococcus gattii* species complex: an international study of wild-type susceptibility endpoint distributions and epidemiological cutoff values for Amphotericin B and flucytosine. Antimicrob Agents Chemother 2012;56 (6):3107–13.
46. **Aller AI, Martin-Mazuelos E, Lozano F, et al.** Correlation of fluconazole MICs with clinical outcome in cryptococcal infection. Antimicrob Agents Chemother 2000;44:1544–8.
47. **Bicanic T.** Symptomatic relapse of HIV-associated cryptococcal meningitis after initial fluconazole monotherapy: the role of

fluconazole resistance and immune reconstitution. Clin Infect Dis 2006;43:1069–73.
48. **Perfect JR, Dismukes WE, Dromer F, et al.** Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. Clin Infect Dis 2010;50:291–322.
49. **Larsen RA, Bauer M, Pitisuttithum P, et al.** Correlation of susceptibility of *Cryptococcus neoformans* to Amphotericin B with clinical outcome. Antmicrob Agents Chemother 2011;55:5624–30.
50. **Verweij PE, Howard SJ, Melchers WJG, Denning DW.** Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. Drug Resist Updat 2009;12:141–7.
51. **Cortez KJ, Roilides E, Quiroz-Telles F, Meletiadis J, Antachopoulos C, et al.** Infections caused by Scedosporium spp. Clin Microbiol Rev 2008;21:157–97.
52. **Nucci M, Anaissie E.** Fusarium infections in immunocompromised patients. Clin Microbiol Rev 2007;20:695–704.
53. **Verweij PE, Mellado E, Melchers WJG.** Multiple-triazole-resistant Aspergillosis. N Engl J Med 2007;356:1481–3.
54. **Baddley JW, Marr KA, Andes DR, et al.** Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the transplant-associated infection surveillance network. J Clin Microbiol 2009;47:3271–5.
55. **Pfaller M, Boyken L, Hollis R, et al.** Use of epidemiological cutoff values to examine 9-year trends in susceptibility of *Aspergillus* species to the triazoles. J Clin Microbiol 2011;49:586–90.
56. **Howard SJ, Cerar D, Anderson MJ, et al.** Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis 2009;15(7):1068–76.
57. **Arendrup MC, Perkhofer S, Howard SJ, et al.** Establishing in vitro-in vivo correlations for *Aspergillus fumigatus*: the challenge of azoles versus echinocandins. Antimicrob Agents Chemother 2008;52:3504–11.
58. **Espinel-Ingroff A, Fothergill A, Fuller J, Johnson E, Pelaez T, Turnidge J.** Wild-type MIC distributions and epidemiological cutoff values for caspofungin and *Aspergillus* spp. for the CLSI broth microdilution method (M38–A2 Document). Antimicrob Agents Chemother 2011;55:2855–9.
59. **Lockhart SR, Zimbeck AJ, Baddley JW, et al.** In vitro echinocandin susceptibility of *Aspergillus* isolates from patients enrolled in the transplant-associated infection surveillance network. Antimicrob Agents Chemother 2011;55:3944–6.
60. **Carver PL, Dang K, Dekarske BM, Rudoni MA, Rutherford D, Zechmeister C.** Evaluation of a unit-specific vs hospital-wide fungal antibiogram for *Candida* species. 17th Symposium on Infections in the Immunocompromised Host (ICHS) Meeting. Genoa, Italy. June 2012.
61. **Shah DN, Yau R, Weston J, et al.** Evaluation of antifungal therapy in patients with candidemia based on susceptibility testing results: implications for antimicrobial stewardship programmes. J Antimicrob Chemother 2011;66:2146–51.