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Antifungal Susceptibility Testing: A Primer for Clinicians

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Clinicians treating patients with fungal infections may turn to susceptibility testing to obtain information regarding the activity of different antifungals against a specific fungus that has been cultured. These results may then be used to make decisions regarding a patient's therapy. However, for many fungal species that are capable of causing invasive infections, clinical breakpoints have not been established. Thus, interpretations of susceptible or resistant cannot be provided by clinical laboratories, and this is especially true for many molds capable of causing severe mycoses. The purpose of this review is to provide an overview of susceptibility testing for clinicians, including the methods used to perform these assays, their limitations, how clinical breakpoints are established, and how the results may be put into context in the absence of interpretive criteria. Examples of when susceptibility testing is not warranted are also provided.

Keywords. antifungal; clinical breakpoint; susceptibility testing.

Over the last several decades, the number of individuals at risk for invasive fungal infections has markedly increased. This has been attributed to the increased number of patients who are immunocompromised, either due to disease or use of immunosuppressive agents, and those in critical care settings. As the number of individuals at risk for fungal infections has grown, so has our recognition of the number of different fungi that are capable of causing disease in humans. It is estimated that there may be between 1.5 and 5 million different fungal species, and more than 300 are capable of causing infections in humans [1–4]. These numbers will only continue to increase as molecular tools become more widely used for the detection and diagnosis of new fungi and fungal diseases and as the number of at-risk individuals continues to rise.

The discovery of new fungi capable of causing disease in both humans and the development of resistance to currently available drugs have far outpaced the availability of new antifungals. One contemporary example of a newly described fungus is that of *Candida auris*. First described in 2009 [5], this emerging pathogen has quickly spread to multiple continents and has been associated with numerous outbreaks in different institutions [6–8]. Isolates of this species are often found to be resistant to multiple antifungals [6–8], and resistance to all currently available antifungals has also been described in some isolates [7, 9, 10]. Another example is *Aspergillus fumigatus*, a well known mold pathogen for which resistance outside of clinical antifungal exposure is now of increasing concern. Azole-resistant *A fumigatus* was first described in the 1990s in patients with chronic exposure to itraconazole [11, 12]. However, more than a decade ago azole-resistant strains were recovered from patients with invasive aspergillosis but without previous azole exposure, and this was subsequently linked to the use of azole-like compounds in the environment [13–15]. Azole resistance linked to environmental exposure has now been reported in countries around the world [3, 16], as have cases of clinical failures in patients treated with azoles for *Aspergillus* infections caused by resistant isolates [3, 17–19]. Because of the discovery of new pathogenic fungi and the increased threat of antifungal resistance, clinicians often use susceptibility testing to help guide therapy in patients with invasive mycoses. The objective of this review is to provide an overview of antifungal susceptibility testing against yeasts and molds. This will include discussions of how antifungal susceptibility testing is performed, how the results may be interpreted and used to guide therapy, as well as their limitations.

METHODS FOR ANTIFUNGAL SUSCEPTIBILITY TESTING

In clinical microbiology laboratories, susceptibility testing is still primarily performed by in vitro phenotypic methods, which measure the ability of a particular drug to inhibit the growth of an organism over a range of concentrations. The readout is the minimum inhibitory concentration (MIC), which corresponds to the lowest concentration of the antifungal that inhibits the growth of the organism. Although significant advances have been made in the arena of clinical microbiology, the antifungal susceptibility assays still used share similarities with those methods described by Sir Alexander Fleming [20] in

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1929 to describe the activity of penicillin against *Staphylococcus* and other bacteria, which included agar diffusion and broth dilution testing.

Several different formats are available for antifungal susceptibility testing. Two organizations that establish and standardize such methods are the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and these are often used as the gold standards by which other methods are evaluated. The CLSI and EUCAST assays most often used are broth microdilution methods that utilize 96-well cell culture trays (Figure 1A) [21–23]. Several commercial assays are also now available for antifungal susceptibility testing. One that is widely used in clinical microbiology laboratories due to the ease of endpoint reading is the YeastOne Sensititre assay (Thermo Scientific, TREK Diagnostics). This is a broth microdilution-based assay that includes a colorimetric dye, resazurin (alamarBlue), which is converted to resorufin (dark pink to red in color) by metabolically active fungal cells. When metabolic activity is inhibited, the wells appear blue, and the change from pink/red to blue is the endpoint used to read the MIC in this assay (Figure 1B). Studies have reported excellent agreement between the YeastOne assay and the broth microdilution assays for *Candida* species, with essential agreement (MICs within 2 dilutions) ranging from 95% to 100% and categorical agreement (same classification of results as susceptible or resistant) between 70% to 100% for both the azoles and the echinocandins [24–31]. Others have also noted good agreement for amphotericin B, different azoles, and the echinocandins with broth microdilution methods against *Aspergillus* species, although some variability has also been reported [32–37]. Good agreement (83% to 100%) has also been reported between the YeastOne and CLSI M38 assays for amphotericin B and posaconazole against Mucorales isolates and *Fusarium* species [32, 38], and similarly for voriconazole against *Fusarium* species [39], although a small number of isolates were generally included in these studies. However, others have reported poor agreement between the YeastOne and the CLSI broth microdilution assays for amphotericin B and different azoles against Mucorales isolates [40].

Another format that is available for antifungal susceptibility testing is that of gradient diffusion (Etest, bioMerieux; MTS, Liofilchem). These assays use plastic strips that contain a concentration gradient of a particular agent, which is placed onto the surface of an agar growth plate that has been inoculated with a fungus. The antifungal diffuses from the strip into the agar, and after a period of incubation, the MIC is read as the concentration where the elliptical zone of inhibition intersects with the strip (Figure 1C). Good essential agreement (92% to 96.8%) has been reported between the CLSI and EUCAST broth microdilution methods and gradient diffusion assays against *Candida* [24, 25], with categorical agreement ranging from 80% to 97% between the Etest and CLSI broth microdilution methods in one study for several azoles and 5-flucytosine [24]. These assays have also been used for mold susceptibility testing, where good agreement has been demonstrated for some, but not all, antifungals against different filamentous fungi, primarily *Aspergillus* and *Fusarium* species [41]. However, marked variability has been reported for members of the order Mucorales and *Scedosporium* species [41–43].

An automated format is also available for use in clinical microbiology laboratories that can perform both yeast antifungal susceptibility testing and yeast species identification (Vitek 2; bioMerieux), with good essential agreement (89.3% to 100%) and categorical agreement (92% to 99.5%) between this method and the CLSI broth microdilution assay [25, 44–49]. It is interesting to note that lower categorical agreement has been reported between both the Vitek and YeastOne methods and the CLSI broth microdilution method for fluconazole against *Candida glabrata* isolates [24, 45, 47]. In addition, this method cannot be used for mold susceptibility testing, and the number of antifungals that can currently be tested by this US Food and Drug Administration (FDA) cleared assay is limited (eg, caspofungin, micafungin, fluconazole, voriconazole, and 5-flucytosine) [50]. It is interesting to note that there have been several reports of falsely elevated antifungal MIC results, especially with amphotericin B, against *C auris* when measured by Vitek 2 [9, 51, 52].

Because antifungal susceptibility testing is still performed by phenotypic assays, the turnaround time for results can be delayed for certain fungi due to incubation periods that are required. For *Candida* species, results are read after 24 hours of incubation. For other yeasts, including *Cryptococcus* and *Rhodotorula* species, a 72-hour incubation period is required per CLSI. For most clinically relevant molds the incubation period is 48 hours, but it can range from 24 hours for the Mucorales (eg, *Rhizopus*, *Mucor*, *Cunninghamella*, *Lichtheimia*, among others) up to 96 hours for dermatophytes. In addition, a pure subculture with adequate growth is needed even before the susceptibility assays can be run. Many clinical microbiology laboratories do not perform their own mold susceptibility testing, so these are sent out to references laboratories, which can further delay results being made available to clinicians.

ENDPOINTS FOR ANTIFUNGAL SUSCEPTIBILITY TESTING

For antifungal susceptibility testing, the endpoints (eg, MIC) used to measure in vitro activity is dependent upon both the organism and the antifungal against which the fungus is tested (Table 1). For amphotericin B, MICs against both yeasts and molds are read as the lowest concentration that results in complete inhibition of growth, and this applies for both broth microdilution and gradient diffusion testing. For the azoles and echinocandins against yeasts, the MIC endpoint is read at the

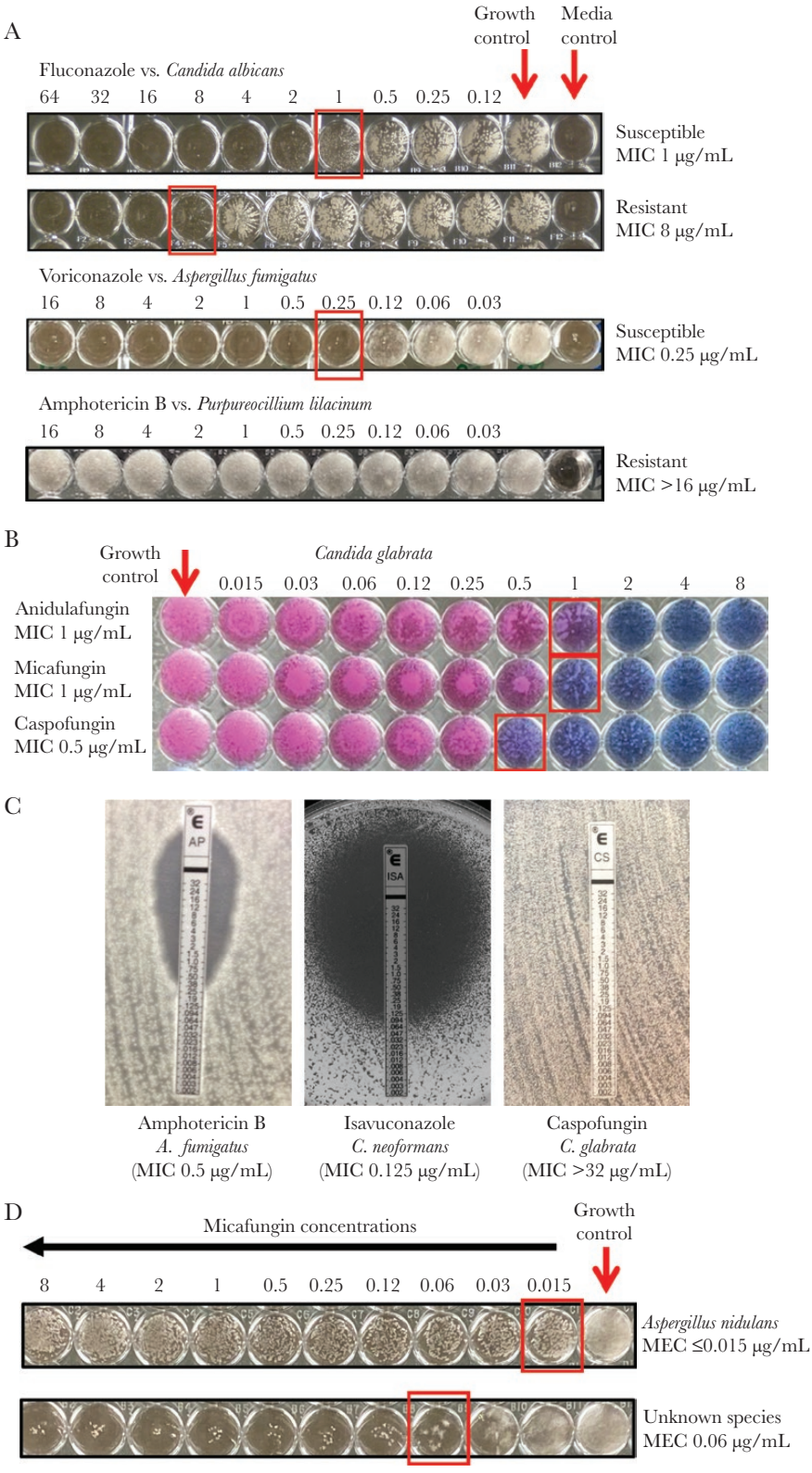

Figure 1. Examples of various phenotypic susceptibility results for yeasts and molds. (A) shows the results of broth microdilution susceptibility testing per the Clinical and Laboratory Standards Institute methodology for fluconazole against *Candida albicans*, voriconazole against *Aspergillus fumigatus*, and amphotericin B against *Purpureocillium lilacinum*. (B) shows susceptibility results as measured by the YeastOne colorimetric assay against *Candida* species. (C) shows susceptibility results as measured by gradient diffusion for amphotericin B against *A fumigatus*, isavuconazole against *Cryptococcus neoformans*, and caspofungin against *Candida glabrata*. (D) shows the minimum effective concentration (MEC) results for micafungin against an *Aspergilllus nidulans* and an unidentified mold isolate. All testing was performed in the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio. Red boxes in A, B, and D indicate minimum inhibitory concentration (MIC)/MEC values.

Table 1. Antifungal Susceptibility Endpoints as Measured by Broth Microdilution

Antifungal Class	Yeasts	Molds	Dermatophytes
Polyenes	100% inhibition of growth		
Azoles	50% inhibition of growth	100% inhibition of growth (50% for fluconazole)	80% inhibition of growth
Allylamines	50% inhibition of growth	100% inhibition of growth	80% inhibition of growth
5-flucytosine	50% inhibition of growth	50% inhibition of growth	50% inhibition of growth
Echinocandins	50% inhibition of growth	MEC	MEC

Abbreviations: MEC, minimum effective concentration (lowest concentration resulting in morphologic changes, such as short, stubby, abnormally branched hyphae).

NOTES: Polyenes - amphotericin B, natamycin, nystatin; Azoles - fluconazole, isavuconazole, itraconazole, posaconazole, voriconazole; Allylamines – terbinafine; Echinocandins - anidulafungin, caspofungin, micafungin.

lowest concentration that results in a 50% inhibition of growth compared to the growth control well.

Against molds, the MIC endpoint for most azoles is read at 100% inhibition of growth, the exception being fluconazole, which is still read as the lowest concentration resulting in 50% inhibition of growth per the CLSI M38 document [23]. For 5-flucytosine, the endpoint for both yeast and molds is 50% inhibition of growth endpoint. Against dermatophytes (ie, *Trichophyton*, *Epidermophyton*, and *Microsporum* species), an 80% inhibition of growth endpoint is recommended for the azoles, terbinafine, and griseofulvin [23]. This endpoint was chosen against dermatophytes because it results in less variability for fungistatic drugs, such as the azoles and terbinafine, due to trailing (ie, small amount of turbidity that may persist even at high concentrations of certain antifungals) [53, 54]. Trailing can also occur with the azoles against *Candida* and other yeasts [55], and for those inexperienced in reading the results of such assays, this can lead to falsely elevated MIC values, and trailing has not been correlated with resistance [56].

With the echinocandins, a different endpoint is used against molds. Here the minimum effective concentration (MEC) is measured, and this corresponds to the lowest concentration that results in morphologic changes (ie, short, stubby hyphae with aberrant branching) (Figure 1D) [57]. The MEC can be considered a mechanism-of-action-based endpoint. The echinocandins inhibit the production of (1,3)-D-glucan polymers within the cell wall of many pathogenic fungi by targeting (1,3)-D-glucan synthase [58]. This enzyme is primarily found at the apical tips and branch points of filamentous fungi and not throughout the hyphae [59, 60]. Thus, exposure of a mold to an echinocandin does not result in growth inhibition but a change in morphology. However, there can be marked intra- and interlaboratory variability with this endpoint. In addition, how well the MEC correlates with clinical outcomes is not well understood.

ANTIFUNGAL CLINICAL BREAKPOINTS

With antimicrobial susceptibility testing, clinicians are often seeking interpretations that classify an organism as either susceptible or resistant. However, such interpretations can only be provided if a clinical breakpoint has been established. A clinical breakpoint is a threshold MIC value, below which a particular organism is considered to be susceptible to the drug being testing, and above which the organism is considered to be resistant. The CLSI interpretive criteria for fluconazole against *Candida* species include susceptible (S), susceptible dose-dependent (SDD), and resistant (S). The SDD category for fluconazole means that higher doses are needed against infections caused by isolates that fall within this category to achieve clinical success [22, 61]. For voriconazole and the echinocandins, the interpretive criteria are susceptible, intermediate, and resistance, and the intermediate category is not interchangeable with SDD used for fluconazole. The EUCAST uses similar categories of interpretation, including (1) susceptible to standard-dosing regimen, (2) susceptible increased exposure (similar to the CLSI category of SDD), (3) resistant, and (4) area of technical uncertainty, which is applied when results fall within an area where reproducible interpretation cannot be achieved [62, 63].

It is important to remember that clinical breakpoints are not naturally occurring phenomena, but instead they are set by committees, including CLSI, EUCAST, and regulatory agencies such as the FDA. Several factors are taken into consideration when breakpoints are being considered. These include MIC distributions, epidemiologic cutoff values ([ECVs], which will be discussed in greater detail later), the pharmacokinetics of the antimicrobial agents and concentrations that are achieved with clinically relevant doses, pharmacokinetic/pharmacodynamic parameters that predict success in in vitro and in vivo studies, and results from clinical studies correlating clinical responses or failures with different MIC values [64, 65]. Clinical breakpoints are subject to review and revision pending the availability of new information, and both CLSI and EUCAST have made changes to antifungal clinical breakpoints. In 2012, CLSI made major changes to their azole and echinocandin antifungal clinical breakpoints against *Candida* species, making them both species- and antifungal-specific. Previously, azole breakpoints had been against *Candida* in general and were not species-specific. For the echinocandins, the same breakpoint applied to each member of this antifungal class. However, since 2012, the CLSI azole and echinocandin

breakpoints are dependent upon both the antifungal tested and the specific fungal species [66].

The number of species/antifungal combinations for which CLSI and EUCAST clinical breakpoints have been established is limited. Current CLSI and EUCAST breakpoints against *Candida* species are shown in Tables 2 and 3. The EUCAST has also set breakpoints for several antifungals against different *Aspergillus* species and for amphotericin B against *Cryptococcus neoformans* (Table 3) [61, 67]. In June 2020, CLSI published a breakpoint for voriconazole against *A fumigatus* (Figure 1A) [68]. Because there are differences between the CLSI and EUCAST testing methods, it is not recommended to apply the breakpoints from one group if the MICs were determined using the methods of the other group. For many pathogenic fungi, especially molds, interpretations of susceptibility results cannot be provided because breakpoints have not yet been set.

The results of antifungal susceptibility testing are most useful when there is a correlation between in vitro results and clinical outcomes. However, the results of studies linking the 2 have been mixed. Numerous studies have been conducted, some reporting positive correlations between in vitro susceptibility results for antifungals against *Candida* species, *C neoformans*, and *Aspergillus* species and clinical outcomes in patients with infections caused by these fungi [69–82], whereas others have not [83–91]. A full review of studies that have attempted to find such correlations is beyond the scope of this review, and these have been reviewed in great detail elsewhere [92–96]. It is not surprising that the results of such studies are mixed given the numerous factors that can influence outcomes in patients with

Table 2. CLSI Antifungal Breakpoints against *Candida* species and *Aspergillus fumigatus*

	Breakpoint			
Organism/Antifungal	Susceptible	SDD	Intermediate	Resistant
Candida albicans				
Anidulafungin	≤0.25	–	0.5	≥1
Caspofungin	≤0.25	–	0.5	≥1
Micafungin	≤0.25	–	0.5	≥1
Fluconazole	≤2	4	–	≥8
Voriconazole	≤0.12	–	0.25–0.5	≥1
Candida glabrata				
Anidulafungin	≤0.12	–	0.25	≥0.5
Caspofungin	≤0.12	–	0.25	≥0.5
Micafungin	≤0.06	–	0.125	≥0.25
Fluconazole	–	≤32	–	≥64
Candida guilliermondii				
Anidulafungin	≤2	–	4	≥8
Caspofungin	≤2	–	4	≥8
Micafungin	≤2	–	4	≥8
Candida krusei				
Anidulafungin	≤0.25	–	0.5	≥1
Caspofungin	≤0.25	–	0.5	≥1
Micafungin	≤0.25	–	0.5	≥1
Fluconazole	–	–	–	–
Voriconazole	≤0.5	–	1	≥2
Candida parapsilosis				
Anidulafungin	≤2	–	4	≥8
Caspofungin	≤2	–	4	≥8
Micafungin	≤2	–	4	≥8
Fluconazole	≤2	4	–	≥8
Voriconazole	≤0.12	–	0.025–0.5	≥1
Candida tropicalis				
Anidulafungin	≤0.25	–	0.5	≥1
Caspofungin	≤0.25	–	0.5	≥1
Micafungin	≤0.25	–	0.5	≥1
Fluconazole	≤2	4	–	≥8
Voriconazole	≤0.12	–	0.25–0.5	≥1
Aspergillus fumigatus				
Voriconazole	≤0.5	–	1	≥2

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; SDD, susceptible dose-dependent.

Table 3. EUCAST Antifungal Breakpoints Against *Candida* and *Aspergillus* Species

Organism/Antifungal	Susceptible	Intermediate	Resistant	ATU
Candida albicans				
Amphotericin B	≤1	–	>1	–
Anidulafungin	≤0.03	–	>0.03	–
Micafungin	≤0.016	–	>0.016	0.03
Fluconazole	≤2	4	>4	–
Itraconazole	≤0.06	–	>0.06	–
Posaconazole	≤0.06	–	>0.06	–
Voriconazole	≤0.06	0.125–0.25	>0.25	–
Candida dubliniensis				
Amphotericin B	≤1	–	>1	–
Fluconazole	≤2	4	>4	–
Itraconazole	≤0.06	–	>0.06	–
Posaconazole	≤0.06	–	>0.06	–
Voriconazole	≤0.06	0.125–0.25	>0.25	–
Candida glabrata				
Amphotericin B	≤1	–	>1	–
Anidulafungin	≤0.06	–	>0.06	–
Micafungin	≤0.03	–	>0.03	–
Fluconazole	–	≤16	>16	–
Candida krusei				
Amphotericin B	≤1	–	>1	–
Anidulafungin	≤0.06	–	>0.06	–
Candida parapsilosis				
Amphotericin B	≤1	–	>1	–
Anidulafungin	≤4	–	>4	–
Micafungin	≤2	–	>2	–
Fluconazole	≤2	4	>4	–
Itraconazole	≤0.125	–	>0.125	–
Posaconazole	≤0.06	–	>0.06	–
Voriconazole	≤0.125	0.25	>0.25	–
Candida tropicalis				
Amphotericin B	≤1	–	>1	–
Anidulafungin	≤0.06	–	>0.06	–
Fluconazole	≤2	4	>4	–
Itraconazole	≤0.125	–	>0.125	–
Posaconazole	≤0.06	–	>0.06	–
Voriconazole	≤0.125	0.25	>0.25	–
Cryptococcus neoformans				
Amphotericin B	≤1	–	>1	–
Aspergillus flavus				
Isavuconazole	≤1	–	>2	2
Itraconazole	≤1	–	>1	2
Aspergillus fumigatus				
Amphotericin B	≤1	–	>1	–
Isavuconazole	≤1	–	>2	2
Itraconazole	≤1	–	>1	2
Posaconazole	≤0.125	–	>0.25	0.25
Voriconazole	≤1	–	>1	2
Aspergillus nidulans				
Isavuconazole	≤0.25	–	>0.25	–
Itraconazole	≤1	–	>1	2
Voriconazole	≤1	–	>1	2
Aspergillus niger				
Amphotericin B	≤1	–	>1	–
Aspergillus terreus				
Isavuconazole	≤1	–	>1	–
Itraconazole	≤1	–	>1	2
Posaconazole	≤0.125	–	>0.25	0.25

Abbreviations: ATU, Area of Technical Uncertainty; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

fungal infections. Many of these studies were retrospective in nature and included a limited number of patients. In addition, for susceptibility testing to be performed, an organism must be cultured, and the sensitivity of fungal cultures for the diagnosis of many invasive fungal infections is limited [97–99], especially for invasive mold infections. Thus, an MIC and its interpretation should not be the sole basis for treatment decisions in patients with fungal infections. Instead, these results should be used in the context of other factors that can influence clinical outcomes.

EPIDEMIOLOGIC CUTOFF VALUES

In the absence of clinical breakpoints, epidemiologic cutoff values (ECVs or ECOFFs) are sometimes available and may provide guidance to clinicians. These are MIC thresholds for a given antifungal that are used to discriminate between wild-type and nonwild-type strains of a specific species, and they are used to identify isolates that may have acquired resistance to a particular antifungal and thus may be less likely to respond to therapy [100–102]. The ECVs are derived statistically and in general encompass 97.5% of isolates within a wild-type MIC distribution. Both CLSI and EUCAST have published ECVs for several different fungal species/antifungal combinations derived from data acquired by different laboratories using their broth microdilution methods [103, 104], and these organizations are actively working to publish new ECVs. The ECVs that have been derived using non-CLSI or EUCAST broth microdilution methods, such as the YeastOne colorimetric assay and gradient diffusion assays, have also been published [105–108]. In a practical sense, in the absence of breakpoints clinicians can use ECVs to make decisions regarding the treatment of infections caused by species known to develop antifungal resistance. Although having an isolate with a wild-type MIC does not guarantee a clinical response, having an isolate with an MIC above the ECV may indicate that resistance has developed, and it may be prudent to use a different antifungal [102]. However, ECVs are not substitutes for clinical breakpoints, and they should not be used in lieu of clinical breakpoints when these are available [102, 103]. In addition, ECVs are still only available for a limited number of fungal species/antifungal combinations, and not all institutions have adopted their use and routinely report ECV interpretations (eg, wild-type vs nonwild-type). In addition, ECVs are not always able to be calculated for a specific antifungal against a specific species due to truncation of the MIC values either at the low or high end of the concentration ranges that are tested. However, knowing the MIC distribution, even with truncated values, may be useful to the clinicians because this allows them to determine whether an antifungal concentration, bloodstream or tissue, above the potential MIC may or may not be achievable [56].

INTRINSIC RESISTANCE AND OTHER EXAMPLES OF WHEN SUSCEPTIBILITY TESTING MAY NOT BE WARRANTED

There are certain circumstances in which susceptibility testing is not warranted, including when intrinsic resistance is present. Intrinsic resistance is defined as inherent or innate, not acquired, antimicrobial resistance, and is reflected by high MIC values in a wild-type population for all or most all representatives of a species against a given antimicrobial agent. Per the CLSI M100 document, a 3% cutoff is used to define intrinsic resistance [109]. In other words, intrinsic resistance is defined when 97% or more of isolates of a particular species are not inhibited at or near the highest concentration of a drug when tested in vitro. Thus, susceptibility testing is unnecessary because resistance is so common. One example of intrinsic antifungal resistance that most clinicians may be aware of is *Candida krusei* and fluconazole. Per the CLSI M60 document, *C krusei* is considered to be intrinsically resistant to fluconazole and susceptibility testing is not warranted [61]. However, several other groups of fungi can demonstrate resistance to particular antifungal classes. For the echinocandins, these include *Cryptococcus*, *Rhodotorula*, and *Trichosporon* species [110–118]. Intrinsic resistance is also found in *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) against amphotericin B (Figure 1A) and the other polyenes, such as nystatin and natamycin [119–122], and *Rasamsonia* species (formerly *Geosmithia* and often misidentified phenotypically as *Paecilomyces* or *Penicillium*) [123, 124] against voriconazole, isavuconazole, and itraconazole [123–126]. Clinical failures have been reported in instances when infections caused by these fungi have been treated with antifungals against which they are intrinsically resistant [123, 127]. Other examples of intrinsic antifungal resistance can be found in Table 4.

However, for some organism/drug combinations, the definition of intrinsic resistance is not met, but recommendations still exist against antifungal testing and using certain antifungals for the treatment of infections caused by specific fungi. For amphotericin B this includes *Candida lusitaniae* and *Aspergillus terreus*. In *C lusitaniae* both intrinsic resistance and resistance that is acquired after clinical exposure to amphotericin B have

Table 4. Examples of Intrinsic *In Vitro* Antifungal Resistance

Antifungal Agent	Genus or Species
Echinocandins	*Cryptococcus, Rhodotorula,* and *Trichosporon* species, Mucorales
Fluconazole	*Candida krusei, Rhodotorula* species, *Aspergillus* species, *Lomentospora prolificans,* Mucorales
Voriconazole	Mucorales, *Rasamsonia* species
Polyenes	*Lomentospora prolificans, Purpureocillium lilacinum*

NOTES: Echinocandins - anidulafungin, caspofungin, micafungin; Polyenes - amphotericin B, natamycin, nystatin.

been reported [128–131]. This in vitro resistance has been observed by different methods of susceptibility testing, although it may be easier to detect by gradient diffusion testing compared with broth microdilution methods [132]. In one study, mutational frequencies for *C lusitaniae* at clinically relevant amphotericin B concentrations were higher than those observed for reference strains of *Candida albicans* and *C glabrata* (8×10^5 vs $<1 \times 10^9$, respectively) [133], while others have reported that *C lusitaniae* is capable of switching between amphotericin B-susceptible and -resistant phenotypes, with some lineages demonstrating stable amphotericin resistance, whereas others were capable of switching between these phenotypes [134]. Thus, amphotericin B susceptibility results against *C lusitaniae* may not be meaningful in helping to guide therapy. Against *A terreus*, amphotericin B MICs are typically elevated (eg, >1 g/mL) [135–140]. However, several studies have also reported lower values against some strains [136, 137, 139, 140], with MICs as low as 0.125 g/mL in one large surveillance study [140]. In surveillance studies, the frequencies of amphotericin B MIC values of <1 g/mL have ranged between 6.8% and 32% [136, 140]. Thus, *A terreus* does not meet the definition of being intrinsically resistant to amphotericin B. However, amphotericin B is not recommended for the treatment of invasive aspergillosis caused by *A terreus* due to poor outcomes that have been reported in clinical studies [141, 142]. It is interesting to note that morphologic heterogeneity has been observed in amphotericin B-resistant *A terreus* cultures [143]. This phenomenon presents as the formation of different sectors that can be detected visually when an amphotericin B-resistant isolate is plated onto drug-free medium. Cultures taken from sectors that appear to be less pigmented, with reduced sporulation, and more cotton-like growth on the agar surface have been reported to have markedly lower amphotericin B MICs compared with resistant isolates (2 g/mL vs 32 g/mL per 1 report) and were also more virulent in a *Galleria mellonella* infection model [143]. In addition, the recently published European Confederation of Medical Mycology/International Society for Human and Animal Mycology/American Society for Microbiology guidelines for the diagnosis and management of rare mold infections does not recommend susceptibility testing for the purpose of decision making for individual patients with currently available antifungals against *Fusarium* species [144], because there has been a lack of correlation between high MIC results and clinical failures [145].

ISSUES ASSOCIATED WITH SUSCEPTIBILITY TESTING OF SPECIFIC ANTIFUNGALS

There are several known methodological issues with currently used antifungal susceptibility assays. For example, interlaboratory variability is an issue with caspofungin testing against *Candida* species when using either the CLSI or EUCAST broth microdilution methods, and this can result in some laboratories reporting caspofungin MIC values that are elevated compared with those for anidulafungin and micafungin [146]. This variability may not be limited to the CLSI and EUCAST methods, because it was also observed in one multilaboratory study in which the YeastOne colorimetric assay was used [147]. Because of this issue, and the chance of a major error occurring (ie, reporting an isolate as resistant when it is susceptible), EUCAST recommends against performing susceptibility testing with caspofungin against yeast, and instead the MIC results of anidulafungin and micafungin serve as surrogate markers for susceptibility or resistance to caspofungin [148, 149]. Although CLSI does not recommend against the use of caspofungin susceptibility testing, it does state that laboratories should confirm caspofungin intermediate or resistant results by either performing testing with anidulafungin or micafungin or by performing deoxyribonucleic acid sequence analysis of the *FKS* genes to identify mutations associated with echinocandin resistance in hotspot regions [61]. Both anidulafungin and micafungin susceptibility testing by the CLSI broth microdilution method have been shown to correlate well with predicting the *FKS* status of isolates, and MIC values for these echinocandins can be used as surrogates for caspofungin susceptibility or resistance [150–152].

Another issue that can occur with in vitro testing of the echinocandins is the paradoxical effect, also known as the Eagle-like effect. This is an attenuation of activity that occurs at higher echinocandin concentrations (growth observed) despite activity being observed at lower levels (no growth observed). First reported by Hall et al [153] with the investigational agent echinocandin cilofungin, it is analogous to the Eagle effect observed with cell wall active antibacterial agents, including penicillin, by Eagle and Musselman in 1948 [154, 155]. The paradoxical effect has been reported for each of the echinocandins against different *Candida* species, including *C auris* most recently [156–159]. However, it is primarily an in vitro phenomenon and is not correlated with in vivo resistance [56, 157, 160].

With amphotericin B, a clustering of MIC values between 0.25 and 1 g/mL is known to occur in broth microdilution formats that use Roswell Park Memorial Institute (RPMI) medium as the growth medium [56]. Because of this, it may be difficult to detect elevated amphotericin B MICs. To overcome this, gradient diffusion assays may be used [161–163]. However, frank amphotericin B resistance is easily observed against some fungi, such as *P lilacinum*, for which MIC values are often at or above the highest concentration of this polyene tested (eg, >16 g/mL) [119–122]. It is also known that the pH of the growth medium may influence in vitro susceptibility results. Several studies have recently reported increases in azole MIC values against *Candida* species when the pH of the growth medium in broth microdilution assays is dropped from a neutral (pH 7) to an acidic value (<5) [164, 165], and the authors have postulated that this may be a reason for azole clinical failures in

the treatment of vulvovaginal candidiasis despite susceptibility testing, which is routinely performed at pH 7 per CLSI and EUCAST methods, that suggest these antifungals should have activity. It is interesting to note that this change in pH does not seem to affect the activity of ibrexafungerp, [166] a new orally available antifungal (triterpenoid class) recently approved for the treatment of vulvovaginal candidiasis in the United States. In contrast, a drop in pH may have the opposite effect on the activity of 5-flucytosine, because studies have demonstrated improved in vitro activity for this agent against *Aspergillus* species in an acidic environment [167, 168], and this may be due to derepression of the *fcyB* gene, which encodes purine-cytosine permease orthologous to known flucytosine importers, at a lower pH [169].

FUTURE DIRECTIONS OF RESISTANCE DETECTION AND CONCLUSIONS

As previously noted, antifungal susceptibility testing for clinical diagnostics is still primarily performed by phenotypic means. Efforts are now being devoted to the development of molecular assays that can be used to detect mechanisms of antifungal resistance, and several different technologies have been evaluated, including those that are culture independent [92, 170]. However, such assays are only useful when the mechanisms of resistance are known and clinically validated. For the echinocandins, point mutations within highly conserved regions (hot spots) of the genes (ie, *FKS1* and *FKS2*) that encode the (1,3)-D-glucan synthase enzyme can cause resistance [171, 172]. Several molecular assays have been described for the detection of point mutations within these hot spot regions, including those that involved real-time multiplexed molecular beacon probes, melt-curve analysis, and microsphere-based assays with asymmetric polymerase chain reaction [173–176]. However, these assays are not widely available in clinical microbiology laboratories. Molecular-based assays have also been developed for the detection of mutations in *CYP51A*, the gene that encodes lanosterol 14-demethylase in *Aspergillus*, the target of the azoles, which confer azole resistance in *A fumigatus*, including a commercial test that detects TR_{34}/L98H and TR_{46}/Y121F/T289A associated with environmental exposure to azoles in direct specimens [177, 178]. Unfortunately, this assay is not FDA cleared for clinical diagnostic use in the United States, and in many phenotypically resistant *A fumigatus* isolates the mechanisms of azole resistance are unknown, because *CYP51A* mutations have not always been detected [3, 18, 179]. In *Candida* species, resistance can develop with azole exposure due to a multitude of mechanisms, including alterations or overexpression of *ERG11*, the gene that encodes lanosterol 14-demethylase in *Candida*, efflux pumps, cellular response factors, as well as genomic plasticity, such as loss of heterozygosity, aneuploidy, and isochromosome formation [180, 181]. The number of mechanisms associated with azole resistance has limited the ability to use molecular assays for the detection of resistance in clinical microbiology [170]. Finally, although our understanding of the mechanisms of antifungal resistance is increasing, major knowledge gaps still exist for many pathogenic fungi. Thus, despite their limitations, phenotypic methods for antifungal susceptibility testing most likely will be used for clinical diagnostics for the foreseeable future.

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