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Usefulness of Antifungal Reference In Vitro Susceptibility Tests as a Guide in Therapeutic Management

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Abstract

Purpose of Review This review provides information on the utility of reference antifungal susceptibility testing methods in the clinical setting.

Recent Findings Clinical and Laboratory Standards Institute (CLSI)/European Committee for Antimicrobial Susceptibility Testing breakpoints (BPs) as predictors of therapy response (reported as either "cured" or "failure") and epidemiological cutoff endpoints (ECVs/ECOFFS) of mutants (harboring specific resistance mechanisms) have been established.

Summary Although ECVs are available for other species and agents and for commercial methods, only reference triazole and echinocandin BPs have been established. Therefore, correlations of in vitro/in vivo results in this review were based on BPs or ECVs for *Candida* spp. and/or *Aspergillus fumigatus*. We also included CLSI ECVs for the *Cryptococcus neoformans* complex and tentative values for *Candida auris*. Overall, BPs/ECVs appear to be useful, but most available data are for correlations between BPs and minimal inhibitory concentrations (MICs) for susceptible isolates. Although ECVs can discriminate between MICs for WT (wild type) and mutants (non-WT), an MIC overlap could be present.

Keywords Antifungal reference methods · Clinical breakpoint for reference methods · ECVs for reference methods · Clinical utility of reference triazole and echinocandin BPs · Clinical utility of reference ECVs

Introduction

Invasive candidiasis, including candidemia, is mostly caused by *Candida albicans, C. glabrata*, *C. parapsilosis, C. tropicalis*, and *C. krusei* and the most common severe mold infections by *Aspergillus fumigatus, A. flavus, A. niger*, and *A. terreus*. *Candida* and *Aspergillus* infections are seen worldwide among immunocompromised as well as non-immunocompromised patients [1•, 2, 3•, 4]. Irrespective of the species, these infections are associated with high mortality and morbidity rates [1•, 2, 3•, 4]. The overall mortality among patients with invasive candidiasis could be ~32% and the rate of treatment success ~67.4% (including treatment with licensed agents up to 2012) [5]. However, the mortality rates could be higher among neutropenic patients or patients infected with non-*Candida albicans* infections such as *C. krusei* and the emerging *C. auris* [6]. A similar mortality rate has been reported for *Aspergillus* infections (47%) that is dependent on both the patient population and age [1•, 2, 3•, 4]. Several antifungal agents have been licensed for the systemic treatment of invasive fungal infections caused by *Candida*, *Cryptococcus*, and/or *Aspergillus* (amphotericin B formulations, flucytosine, triazoles, including isavuconazole, and three echinocandins) and others are under investigation [7••, 8••, 9].

The overall utility of antimicrobial susceptibility testing is to aid in selecting the most effective agent for the treatment of microbial infections, which is best achieved by species-specific categorical/interpretive end points, breakpoints (BPs), or epidemiological cutoff values (ECVs). Although both standard and commercial antifungal susceptibility testing methods are available for evaluating the infecting isolates, BPs are only

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established for a few fungal species and agent combinations. More recently and based solely on MIC (minimal inhibitory concentration) or MEC (minimal effective concentration) distributions, method-specific ECVs or ECOFFs were defined for a variety of yeast and mold species for reference and commercial methods. However, very little information is found in the literature regarding specific correlations of clinical outcome and antifungal susceptibility testing results using BPs or ECVs. One of the reasons is that the infecting agent is not always available due to the lack of reliable methods for the diagnosis of invasive fungal infections. Blood cultures are unreliable markers of candidemia, and in the case of aspergillosis, the *Aspergillus* PCR assay is substantially more sensitive (77–84%) than blood and respiratory fluids cultures [8••].

Earlier reviews focused on the different available methodologies for antifungal susceptibility testing as well as the expected in vitro results for a variety of fungal species. We present the correlations of MICs with either the clinical outcome to therapy or the presence of genetic resistance mechanisms in the infecting isolate. The literature was first searched for (i) the value of established reference triazole or echinocandin BPs as predictors of clinical response to therapy (reported as either "cured" or "failure") and (ii) the value of reference ECVs as predictors of mutant isolates when specific resistant mechanism among *Candida* or *Aspergillus* isolates were present. Given that ECVs for the *Cryptococcus neoformans* complex as well as tentative values for *C. auris* were recently published, those combinations were also included.

Reference Antifungal Susceptibility Methods and Categorical/Interpretive End points

CLSI The Clinical and Laboratory Standards Institute (CLSI) has developed broth microdilution susceptibility testing methods for *Candida* spp., the *C. neoformans* complex, and molds (M27-4th ed. and M38-3rd ed.) documents, respectively) [10, 11]. CLSI BPs were established for most common *Candida* spp. for MIC categorization of triazoles and echinocandins taking into account (i) the MIC, (ii) resistance mechanisms, (iii) PK/PD (pharmacokinetic/pharmacodynamic) information, and (iv) patient response to therapy from clinical trials (Tables 1 and 4) [12••, 13–21]. CLSI method-dependent triazole (isavuconazole included) and echinocandin ECVs also have been defined for species of *Candida*, *Aspergillus, C. neoformans*, and other molds [22–26, 27••, 28•, 29], including some species for which BPs have not been established [28•]. The calculation of CLSI ECVs met the criteria postulated by this organization: a minimum of 100 MIC values in the particular pool of data points for ECV definition analysis, modal compatibility among at least three contributing laboratories, and five data points in each distribution/species/agent/laboratory [29]. The ECV calculation should take into account the inherent variability of the test (usually within 1 doubling dilution). The ECVs listed in Table 4 were calculated by the iterative ECOFFinder technique and encompassed 95 to 97.5% of the isolates [30]. Approved CLSI categorical end points are listed in CLSI documents M60 [12••], M59 (approved ECVs) [27••], and elsewhere [28•].

There are some important considerations when using the CLSI M27 method and BPs [12••]. The CLSI BPs for voriconazole and *C. krusei* are based on the clinical response to voriconazole from 7 of 9 (78%) patients evaluated during voriconazole clinical trials (data not included in Tables 1 and 4). Since *C. krusei* is innately resistant to fluconazole, testing for this agent/species is not needed. Given that the clinical outcome among patients treated with either fluconazole and especially voriconazole for *C. glabrata* infections was poor regardless of the MIC endpoint, only the fluconazole susceptible-dose dependent (SDD: ≤ 32 μg/ml) and resistant (≥ 64 μg/ml) endpoints are documented in the CLSI M60 document [12••]. Because of that, this document states that for *C. glabrata* infections versus fluconazole, higher doses than the standard 6 mg/kg/day may be required for adults.

More importantly, poor interlaboratory agreement of caspofungin MICs for *Candida* spp. has been reported for both CLSI and European Committee for Antimicrobial Susceptibility Testing (EUCAST) MICs [31]. However, the CLSI M60 lists BPs for caspofungin and *Candida* spp. with the recommendation that caspofungin "susceptible MICs" should be confirmed with follow-up anidulafungin and micafungin MICs and/or the presence of mutations in the infecting isolate [12••]. Neither step is practical or feasible in most clinical laboratories as well as being time consuming which will further delay the report. The best approach would be to use either anidulafungin or micafungin as surrogate indicators of echinocandin resistance, since CLSI BPs and ECVs are available for *Candida* spp. and those two agents. Because of that, Table 4 only provides anidulafungin data as the surrogate indicator.

EUCAST European Committee for Antimicrobial Susceptibility Testing Broth microdilution methods were also developed by the EUCAST for testing *Candida* and *Aspergillus* spp. The EUCAST BPs are based on MIC distributions, PK/PD information, correlation and regression trees (CART) analysis, including Monte Carlo simulations, and to a certain extent clinical data (http://www.eucast.org/ast_fungi/) [32–35]. Although susceptible EUCAST and CLSI fluconazole and voriconazole BPs are the same for *C. albicans*, *C. parapsilosis*, and *C. tropicalis* (fluconazole BPs < 2 μg/ml and voriconazole BPs $<$ 0.12 μg/ml) (Table 1), there are some substantial differences among other CLSI and EUCAST BPs [35]. In addition to the susceptible and resistant breakpoints, an intermediate BP is available for fluconazole (not usually listed). The intermedia EUCAST BP is "the value between the S and R categories" [34]. EUCAST has developed BPs for the triazoles, including

Table 1 Correlation of CLSI and EUCAST MICs in micrograms per millilitre and patient outcome according to BPs for *Candida albicans* during clinical trials

No. of patients cured/no. of patients treated (%)						
Fluconazole			Voriconazole		Anidulafungin	
CLSI/EUC BPs[2]	CLSI data[1]	EUCAST data[2]	CLSI BPs[2]	CLSI data[1]	CLSI BPs[1]	CLSI data[1]
≤ 2/≤ 2 (S)	320/339 (94)	136/145 (94)	≤ 0.12 (I)	60/82 (74)	≤ 0.25 (S)	112/121 (93)
4 (SDD)/I[3]	21/23 (91)	8/12 (67)[3]	0.25–0.5 (I)[2]	6/6 (NA)	0.25 (1)[2]	No data
≥ 8 (R)/> 4	32/57 (56)	12/101 (12)	≥ 1 (R)	3/7 (NA)	≥ 1 (R)	No data

NA too few cases for a meaningful percent

[1] Clinical response mostly from non-neutropenic patients and adapted from refs. [15, 17, 21, 32, 33]

[2] S, susceptible; I, intermediate; R, resistant; SDD, susceptible dose-dependent (CLSI fluconazole) [12••]

[3] EUCAST susceptible and resistant BPs ≤ 2 and > 4 μg/ml. http://www.eucast.org/ast_of_fungi/rationale_documents_for_antifungals/

isavuconazole and amphotericin B for *A. fumigatus* and other *Aspergillus* spp. and several ECOFFs for *Candida*/*Aspergillus* spp., including tentative ECVs for *C. auris* and most available agents [36] (http://www.eucast.org/documents/consultations/). EUCAST guidelines for setting ECVs are in the "EUCAST discussion document" (under review). But it is not clear yet what are the restrictions or criteria regarding the MIC data used to define EUCAST ECVs, but the listed "combined distributions are from multiple sources and time periods" when ECVs/BPs were established for the particular agent. Overall, EUCAST ECOFFs are set "when the visual and statistical ECOFFs are no more than one 2-fold dilution apart" [36] (http://www.eucast.org/documents/consultations/), (www.eucast.org/clinical_breakpoints). Interlaboratory discrepancies were also evident among caspofungin EUCAST MICs for *Candida* spp. [31]. The recommendation is to use either anidulafungin or micafungin as surrogate data (www.eucast.org/clinical_breakpoints).

Commercial Etest and SYO Methods Although it is not the scope of the present review to cover the commercial methods, ECVs have been developed for the widely used Sensititre Yeast One (SYO) and Etest methods [37–41, 42••, 43••]. However, the lack of suitable data has precluded the establishment of BPs for the categorical interpretation of MICs by the commercial methods. Presently, these two methods rely on CLSI interpretive categories as well quality control (QC) data. Also, despite favorable comparisons between MICs by commercial and reference methods to evaluate method equivalence before marketing, substantial method-dependent differences were reported between CLSI, SYO, and Etest ECVs [40, 43••]. Therefore, it is not advisable to use reference BPs/ECVs to categorize MICs that are obtained by either of these two methods. Instead, the physician should be apprised regarding the potential role/usefulness of the ECV as a predictor of antifungal resistance [28•,29]. Recent ECV studies have concluded that, although both SYO and Etest methods are easier to perform and more practical than reference methods, due to interlaboratory discrepancies among the participant laboratories, the SYO method provides more reliable data for *Candida* spp. and the Etest for *Aspergillus* spp. [42••, 43••].

Resistance Mechanisms in Fungal Isolates

The impact of azole and echinocandin resistance in MIC data is widely recognized, and various mutational mechanisms of resistance have been elucidated, especially for the most prevalent *Candida* spp. and *A. fumigatus* [15, 17, 22, 23, 42••, 43••, 44••, 45–47, 48••, 49–53]. The use of triazole therapies has led to in vitro resistance among *Candida* and other yeasts to fluconazole, voriconazole and posaconazole [15, 17, 22, 44••, 45–47, 48••]. The azoles block the pathway of ergosterol biosynthesis by inhibiting the 14-α-lanosterol demethylase enzyme. Various molecular mechanisms are associated with in vitro resistance to the triazoles and *Candida* spp., such as modifications in the quality or quantity of the target enzyme (*ERG11* gene mutations/modifications and/or overexpression) or the *MRR1* transcriptional regulator [15, 17, 44••, 45–47, 48••]. Also, the active azole efflux from the fungal cell mediated through the activation of multidrug efflux transporters encoded by *CDR1, CDR2*, or *PDR1* genes has been reported [15, 17, 44••, 45–47, 48••]. More recently, resistance to the echinocandins has become prevalent especially among *C. glabrata* and other *Candida* isolates [49–53]. Echinocandin resistance among *Candida* isolates is usually associated with mutations in the 1,3 β-D-glucan synthase target enzyme encoded by either the hot *FKS1* (*Candida* species, including *C. glabrata*) or *FKS2* (*C. glabrata*) gene muations [49–51]. Resistance to the triazoles against *Aspergillus* spp. also has increased in the last decade, especially in Europe. The most frequent resistance mechanisms in *A. fumigatus* are the mutations in the *Cyp51A* gene [28•, 42••]. However, although ECVs have been proposed for other agents, very little data are available regarding the resistance mechanisms for other agents and fungal species [28•].

Role of the Categorical/Interpretive Endpoints

BPs and ECVs As discussed above, the BP is the predictor of clinical outcome guiding the clinician in choosing the best agent for the infecting isolate being evaluated [12••,15, 17, 21]. The ECV, as the new categorical end point, will differentiate the non-WT (strains potentially harboring resistance mechanisms) from the WT isolates (strains without known resistance mechanisms) in the MIC/MEC distribution [22, 23, 28•, 29]. Because ECVs do not take into account the pharmacology of the antifungal agent or the findings from clinical outcome studies, the ECV will not categorize a fungal isolate as susceptible or resistant as BPs do. The ECV is the highest MIC/MEC without phenotypically expressed resistance and is not a predictor of treatment outcome. Due to all those considerations, the ECV only should be used when BPs have not been established for the species/agent being evaluated; they could provide clinical guidance regarding the potential outcome/response to therapy [28•, 29]. Again, regardless of their role, both categorical end points are species/agent dependent as well as method-dependent. ECVs also are particularly useful in tracking the emergence of resistance at an institution. Finally, both categorical end points only are useful in the clinical setting, when the causative fungal isolate is identified to the species level.

Factors That Obscure In Vitro Versus In Vivo Correlations Using BPs or ECVs

Other factors, beside the MIC for the infecting isolate and therapy with the licensed antifungal agents, can obscure the correlations of in vitro versus in vivo results as follows: (i) the extrapolation of method versus categorical end point, (ii) the PK/PD status, (iii) the host immune status, (iv) the site of infection, and (v) the infecting isolate species [54]. In 2012, based on data from several clinical trials for invasive *Candida* infections, the following factors were identified as predictors of patient mortality [5]: (i) increasing age, (ii) the Acute Physiology and Chronic Health Evaluations, (iii) the use of immunosuppressive therapy, and (iv) infections caused by *C. tropicalis* and more recently, among candidemia patients, by the fluconazole resistant *C. auris* (~ 70% mortality) [6]. However, removal of the central venous catheter and/or treatment with an echinocandin instead of amphotericin B or a triazole has been associated with decreased mortality [5, 55]. Tables 2 and 3 depict the impact of the immune status and/or of the infecting isolate species on the clinical response to therapy during earlier clinical trials [56]. Some echinocandin PK/PD factors are discussed in more detail below.

Table 2 Response to antifungal therapy for *Candida* infections according to species during clinical trials

No. of patients with a favorable response/no. of patients in subgroup (%)			
Species	Micafungin	Caspofungin	Amphotericin B
C. albicans	188/249 (76)	23/36 (64)	34/58 (58)
C. glabrata	68/87 (78)	10/13 (77)	8/10 (80)
C. krusei	10/14 (71)	NA	NA
C. parapsilosis	79/112 (71)	14/20 (70)	3/20 (65)
C. tropicalis	70/97 (72)	17/20 (85)	10/14 (71)

Data from refs: [18, 21, 56]

NA not available

Table 3 Response to antifungal therapy for *Candida* infections according to the immune status during clinical trials

No. of patients with a favorable response/no. of patients in subgroup (%)		
	Caspofungin (%)	Amphotericin B (%)
Neutropenic (ANC < 500 ml)	7/14 (50)	4/10 (40)
Non-neutropenic (ANC ≥ 500 ml)	73/95 (77)	67/105 (64)

Data from refs: [18, 21, 56]

NA not available

The echinocandin PD target for 11 *C. glabrata* mutants (*fks* mutations, MICs 0.06–4 μg/ml) and 8 WT (MICs 0.03–0.25 μg/ml) was evaluated using an in vivo invasive candidiasis/echinocandin model [57]. The mean free anidulafungin 24-h AUCf/MIC ratio associated with stasis for the WT group was 13.2 and 3.43 for the mutants. In patients, the PD target was achieved when the anidulafungin MICs for the infecting isolates was 0.25 μg/ml (*C. glabrata* infections). The conclusion was that the MIC could be a better predictor of the outcome of therapy than the identification of genetic mutations [57]. Regarding the PK/PD status in patients treated for invasive candidiasis with micafungin (100 mg daily), there was a relationship between a patient's weight and drug exposure which indicated that patients weighing > 100 kg could have insufficient drug exposure and thus potentially inadequate antifungal treatment [58•]; the median micafungin MIC for the infecting isolates was 0.016 μg/ml (susceptible). Therefore, it is possible that patients infected with *Candida* isolates with higher MICs also could have insufficient drug exposure.

In Vitro Versus In Vivo Correlations: the Triazoles Versus *Candida* spp., *C. neoformans* VNI, and *A. fumigatus*

Because reports of response to therapy and their correlation with MICs are scarce, the data from earlier fluconazole and voriconazole and *Candida* spp. clinical trials are included in Table 4 [15, 17]. However, more data were available regarding the presence of mutations and their effect on the MIC end

point. The CLSI triazole MIC data for *Candida* spp. and *A. fumigatus Cyp51A* mutants were pooled from the multicenter studies that defined CLSI ECVs and other reports [42••, 44••, 45–47, 48••, 59–66]. MIC data for mutants validated ECVs as predictors of triazole resistance [24, 28•, 29].

***Candida* spp. Versus the Triazoles** The clinical response to therapy and the presence of specific mutations for the infecting isolate are depicted in Table 4; the overall clinical response according to CLSI (fluconazole and voriconazole) and EUCAST (fluconazole) BPs *C. albicans,* by categories are summarized in Table 1. Clinical trials to assess the efficacy of fluconazole and voriconazole prior to licensing yielded very little data for resistant or other non-*C. albicans* isolates [15, 17, 32]. The most probable reasons were that patients were not infected with non-*C. albicans* isolates as *C. albicans* was and continues being the most prevalent species; also, perhaps it was too early for resistance development. The treatment response data for each species and BP was summarized when the current CLSI BPs were proposed (between 2010 and 2011; Table 4) [12••, 15, 17]. The correlation between fluconazole MICs ≤2 μg/ml for *C. albicans* (susceptible BP) and successful clinical response to therapy (patients considered cured) was 94% and 70% for *C. tropicalis* (smaller number of treated patients with the latter species (20 versus 339) (Table 4). Regarding the fluconazole SDD value for *C. glabrata* of ≤ 32 μg/ml, 40 of 64 (63%) patients responded to therapy. Correlations between voriconazole MICs and clinical outcome for *C. albicans*, *C. parapsilosis* and *C. tropicalis* were similar (72–82%) [17] (total number of patients infected with each of these three species was below 100) (Table 4). Further information about the role of PK/PD and MICs on the clinical outcome of patients treated with triazoles can be found in a recent review [67].

MICs from multiple laboratories enabled the definition of CLSI triazole ECVs for the five *Candida* spp. listed in Table 4, as well as for *C. guilliermondii*, *C. lusitaniae*, and *C. krusei* [22, 27••, 28•]. ECVs for those three species were not listed, because genetic information is not available, but those ECVs can be found elsewhere [22, 28•]. The performance of fluconazole and voriconazole ECVs in recognizing *Candida* spp. mutants was excellent for most of the species/agent combinations listed in Table 4 (92 to 100%) [22, 44••, 45–47]. The same applied among the WT isolates evaluated (MICs equal to or below the ECV). Although CLSI has not defined isavuconazole ECVs for *Candida* spp., the available CLSI data for *C. albicans* and *C. glabrata* mutants are listed in Table 4 [44••]. Insufficient data precluded a proper evaluation of posaconazole ECVs for *Candida* spp. However, with the exception of fluconazole, some "overlap" was observed with the other triazoles. The overlap indicated that MICs for confirmed mutants were below the ECV (false WT) or that MICs for WT isolates (without known mechanism of resistance; false non-WT) were above the ECV. This phenomenon can be observed in most published studies, where data for confirmed mutant and WT isolates have been reported.

Tentative EUCAST/CLSI ECVs for *C. auris* were defined for various agents [36], including fluconazole and voriconazole; data for 42 mutants and 36 WT isolates were recently reported (most common mutations, *EGR11* Y132F and K143R, also found among *C. tropicalis* and *C. parapsilosis* mutants) [48••]. CLSI fluconazole MICs for the mutants were 4–>128 μg/ml (mode, ≥16 μg/ml) and for the WT 2–64 μg/ml (mode, 2 μg/ml), so there was an MIC overlap among WT and mutants. Similar overlap was observed with the voriconazole ECV (8 μg/ml; Table 4) as well as discrepant modes for both agents [36, 48••]. Redefinition of these ECVs should be considered to evaluate interlaboratory modal agreement.

***C. neoformans* VNI Versus Fluconazole** In addition to ECVs for *Candida* and the triazoles, the CLSI has approved ECVs for various *Cryptococcus* spp., including *C. neoformans* VNI, the triazoles, amphotericin B, flucytosine, and isavuconazole [25, 27••, 28•]. The CLSI ECV for *C. neoformans* VNI and isavuconazole is 8 μg/ml (WT values); isavuconazole MICs of ≤8 μg/ml (WT values) for eight isolates were reported from patients treated with this agent for cryptococcal meningitis; six of eight patients had a successful outcome to therapy (Table 4) [68]. It is interesting that fluconazole MICs were 0.25 to 8 μg/ml (presumptively WT isolates) for isolates from patients reported as cured, while five therapeutic failures correlated with MICs of ≥16 mg/ml [69] (fluconazole data not listed in Table 4); these latter MICs were obtained using the yeast nitrogen base instead of the CLSI RPMI broth [10].

***Aspergillus* spp. Versus the Triazoles** The CLSI has not developed BPs for molds due to lack of suitable clinical data, but ECVs have been approved for amphotericin B and the triazoles, including isavuconazole for *A. fumigatus* and other molds [24, 26, 27••, 28•, 42••]. Table 4 provides a summary of the performance of the available triazole ECVs in identifying potential *A. fumigatus* non-WT isolates (data pooled from various published studies) [42••, 59–66]. The CLSI itraconazole ECV of 1 μg/ml (97%) is superior to the posaconazole ECV of 0.25 μg/ml (77%) and both voriconazole and isavuconazole ECVs of 1 μg/m (83/84%, respectively) in identifying the *Cyp51A* mutants. However, certain mutations do not affect posaconazole and voriconazole MICs as they do itraconazole values. It also has been suggested that some mutations must be silent polymorphisms [42••]. A recent clinical report correlated CLSI/EUCAST voriconazole MICs > 8 μg/ml with a patient's treatment failure to this agent; data for pretreatment isolate(s) was not provided [70].

EUCAST ECVs have been established for *Aspergillus* spp. and the triazoles. EUCAST posaconazole MICs were pooled for 52 *A. fumigatus* mutants in a recent collaborative study

Table 4 Correlation of CLSI triazole and echinocandin MICs with either patient outcome to therapy or the presence of genetic mutations

Species	Agent	SBP in μg/ml (no. Pts cured/total)	% Cured	ECV in μg/ml	No. non-WT > ECV/total no. WT ≤ ECV/total	% Non-WT > ECV % WT ≤ ECV	Reported mutation	Reference
C. albicans	FL	≤2 (320/339)	94	0.5	> 0.5 (72/72)	100	*ERG11, MRR 1*/overexpression	15, 22, 44
					≤ 0.5 (29/29)	100	WT	
	POS	NA		0.06	> 0.06 (64/67)	96	*ERG11, MRR 1*/overexpression	22
					≤ 0.06 (21/29)	72	Overlap	
	VOR	≤ 0.12 (60/82)	73	0.03	> 0.03 (44/44)	100	*ERG11, MRR 1*/overexpression	15, 17, 22
					≤ 0.03 (26/29)	90	Overlap	
	ISA	NA		NA	≥ 0.01–8 (22/25)	90	*ERG11, MRR 1*/overexpression	44
					≤ 0.03 (11/11)	100	WT	
C. dubliniensis	FL	NA		0.5	> 0.5 (26/26)	100	*ERG11*/overexpression	22
					≤ 0.5 (12/12)	100	WT	
	POS	NA		0.25	> 0.25 (1/1)	ID	*ERG11*/overexpression	22
					≤ 0.25 (1/2)	ID	WT	
	VOR	NA		0.03	> 0.03 (4/3)	ID	*ERG11*/overexpression	22
					NA	NA	Overlap	
C. glabrata	FLU	≤ 32 (40/64)	63	8	> 8 (66/69)	96	*CgPDR1, CgCDR1*/overlap	15, 22, 44
					≤ 8 (38/38)	100	WT	
	POS	NA		1	> 1 (6/9)	67	*CgPDR1, CgCDR1*/overlap	44
					≤ 1(7/7)	100	Others/overlap	
	ITR	NA		4	> 4 (2/9)	23	Overlap	44
					≤ 4 (7/7)	100	Overlap	
	VOR	NA		0.25	> 0.25 (37/39)	95	*CgPDR1, CgCDR1*/overlap	22, 44
					≤ 0.25 (14/19)	74	Overlap	
	ISA	NA		NA	0.25–1 (7/7)	100	*CgPDR1, CgCDR1*/overlap	44
					0.25–16 (9/9)	100		
C. parapsilosis	FLU	≤ 2 (NA)		1	> 1 (64/64)	100	*ERG11* or overexpression	15, 22, 45–47
					< 1 (5/5)	ID	WT	
	POS	NA		0.25	> 0.25 (3/3)	ID	*ERG11* or overexpression	15, 22, 45–47
					< 0.25 (1/1)	ID	WT	
	VOR	≤ 0.12 (29/34)	82	0.03	> 0.03 (59/64)	92	*ERG11* or overexpression	15, 22, 45–47
					< 0.03 (19/21)	5/5	WT	
C. tropicalis	FLU	≤ 2 (14/20)	70	1	> 1 (3/3)	ID	*ERG11* or overexpression	15, 22
					≤ 1 (1/1)	ID	WT	
	VOR	≤ 0.12 (34/47)	72	0.06	> 0.06 (3/3)	ID	*EG11* Y132F, K143R	15, 22
					≤ 0.0 6 (1/1)	ID	WT	
C. auris	FLU	NA		64*	4– > 128	NA	*EG11* Y132F, K143R/overlap	36, 48
					2–64	NA	Overlap	
	VOR	NA		8*	0.06– ≥ 16	NA	*EG11* Y132F, K143R/overlap	36, 48
					0.03–1	NA	WT	
C. neoformans	ISA	NA		8	≤ 8 (8/8)	6/8 (Cured)	All WT	25, 68
A. fumigatus	ITR	NA		1	> 1(231/238)	97	*cyp51A* mutations/overlap	24, 60, 62, 64, 65
					≤ 2(127/134)	95	Overlap	
	POS	NA		0.25	> 0.5 (245/320)	77	*cyp51A* mutations/overlap	24, 42, 60, 64, 65
					≤ 0.25 (2118/2188)	97	Overlap	
	VOR	NA		1	≥ 2 (196/235)	83	*cyp51A* mutations/overlap*	24, 61, 63, 64
					≤ 1(98/117)	84	Overlap	

Table 4 (continued)

Species	Agent	SBP in μg/ml (no. Pts cured/total)	% Cured	ECV in μg/ml	No. non-WT > ECV/total no. WT ≤ ECV/total	% Non-WT > ECV % WT ≤ ECV	Reported mutation	Reference
	ISA	NA		1	> 2 (31/37)	84	*cyp51A* mutations/overlap	26, 64, 66
					≤ 1 (11/13)	85	Overlap	
C. albicans	AND	≤ 0.25 (112/121)	93	0.12	> 0.12(10/10)	100	*fks1* mutations	21, 23
					≤ 0.12 (41/42)	98	Overlap	
C. glabrata		≤ 0.12 (84/92)	92	0.12	> 0.12 (70/88)	88	*fks1/fks2*mutations/overlap	21, 23, 50–53
					≤ 012 (136/140)	97	Overlap	
C. krusei		≤ 0.25 (3/6)	NA	0.25	> 0.25 (10/11)	91	*fks1* mutations	21, 23, 51
					≤ 0.25(27/28)	96	Overlap	
C. parapsilosis		≤ 2 (10/11)	91	8	NA	NA	NA	21, 23
C. tropicalis		≤ 0.25 (18/21)	86	0.12	> 0.12 (10/11)	91	*Fks2* mutations/overlap	21, 23, 49, 51
		≥ 0.5			≤ 012 (27/28)	96	Overlap	

Clinical data for FLU, VOR, and AND from clinical trials [13, 14, 16, 18–20] and as summarized in the last column references. Most patients were infected with isolates with low MICs. Overlap: the MICs for isolates harboring mutations (non-WT) were either equal or below the ECV

FLU fluconazole, *ITR* itraconazole, *POS* posaconazole, *VOR* voriconazole, *ISA* isavuconazole, *AND* anidulafungin, *SBP* susceptible breakpoint, except for fluconazole and *C. glabrata* (SDD), *ECV/ECOFF* epidemiologic cutoff value, *ID* insufficient data for meaningful percentages, *NA* not available

*CLSI ECVs for this species have not been approved by the CLSI

[42••]; itraconazole and voriconazole data were reported elsewhere [71] (not shown in Table 4). The EUCAST posaconazole ECV of 0.25 μg/ml would have identified as non-WT, 40 of the 52 isolates harboring confirmed mutational changes [42••]. EUCAST triazole ECVs for *A. fumigatus* were first defined in an earlier study, where 32 *A. fumigatus* mutants with itraconazole MICs ≥ 8 μg/ml were evaluated [72]. Posaconazole MICs for those mutants were 0.12–> 8 μg/ml, which indicated a similar overlap between MICs for WT and mutant isolates, but not among itraconazole MICs [71, 72]. Similar mutations were observed in both studies (G54, M220 or TR34), but the latter evaluations included TR46 [42••, 71].

However, it was recently reported that there was no relationship between patient outcome to therapy versus isavuconazole and voriconazole MICs for infecting *Aspergillus* isolates [73]. Their summary was as follows: of 49 patients treated with isavuconazole, 6 patients died (MICs, > 2 μg/ml for 1/6 of the infecting isolates); 43/49 patients responded (alive at the end of therapy) and MICs were ≤ 2 μg/ml for 38/43 (88%) of the infecting isolates (data not listed in Table 4). Although the isavuconazole EUCAST ECV is ≤ 2 μg/ml for *A. fumigatus*, the proposed BP is ≤ 1 μg/ml [34]. In the case of voriconazole, both EUCAST BP and ECV are ≤ 1 μg/ml for *A. fumigatus*, so the overlap between "susceptible" and "resistant" MICs was also evident with this agent. ECVs and BPs are species specific [12••, 28•, 29], but the infecting isolates were reported as "*Aspergillus*," so the proportion of *A. fumigatus* in the set is not clear. However, it appears that the isavuconazole EUCAST BP needs to be adjusted for this species, as more data from clinical trials becomes available. It is interesting that based on an in vitro dynamic model of invasive pulmonary aspergillosis with simulation of human-like voriconazole PK and galactomannan as a biomarker, the trough concentration that achieved suppression of galactomannan was the EUCAST voriconazole MIC of 1.68 μg/ml [63]. The authors suggested that for voriconazole and *A. fumigatus*, the BPs could be ≤ 1 μg/ml and > 2 μg/ml, as susceptible and resistant BPs, respectively.

***Candida* spp. and the Echinocandins** Elevated echinocandin MICs for *Candida* spp. have been associated with amino acid substitutions in the *FKS1* and *FKS2* genes, therapeutic failure, and/or breakthrough infections. However, resistance mechanisms are not as clearly elucidated for *Aspergillus* spp. There is also the interlaboratory variability issue among reference caspofungin MICs for *Candida* spp. [31]. Due to those concerns, Table 4 only depicts the correlation of CLSI anidulafungin MICs with treatment outcome from clinical trials and other report [21, 53]. The correlation of CLSI anidulafungin ECVs and MICs for *Candida* mutants was also provided in Table 4, including the data summarized during the calculation of echinocandin ECVs and from other studies [23,

49–53]. The clinical data for patients treated with micafungin, including data for mutants, is summarized below [52]. Those two echinocandins have been recommended as surrogate agents. Some of these studies were published before ECVs were defined according to the current criteria, so the correlations were based on BPs instead of ECVs for the mutants [51–53]. However, those data were re-evaluated according to current CLSI ECVs [23, 27••, 28•].

The correlation of CLSI MICs for *C. albicans, C. glabrata*, and *C. parapsilosis* and clinical response to anidulafungin therapy was excellent for the susceptible isolates (91–93%) [21, 53]; lower percentages were observed for the other two species, but the number of patients treated was small (Table 4) [21]. Clinical trials did not include much anidulafungin data for resistant or intermediate isolates; in a recent study, anidulafungin MICs > 0.12 μg/ml for *C. glabrata* only predicted 5/22 treatment failures [53]. In a micafungin study [52], 6/16 patients responded to therapy (MICs 0.03–1 μg/ml); however, the micafungin MIC range was 0.12 to 4 μg/ml (only two intermediate values, 0.12 μg/ml) for the 10 treatment failure isolates [52]. Results were similar with either agent in both studies. So, more data are needed to clarify these discrepant results.

Concerning the pooled echinocandin data for mutants and ECVs [23], the predictor value of the anidulafungin CLSI ECVs in detecting mutants ranged from 88% (*C. glabrata*) to > 90% for the other *Candida* species (Table 4). The amount of data was small for some of these species/anidulafungin combinations.

Using the EUCAST method, MIC data for 20 *Candida* spp. (8 *C. albicans*, 2 *C. krusei*, and 10 *C. glabrata*) harboring *FKS1* or *FKS2* mutations indicated that anidulafungin MICs for these three species were above the EUCAST ECVs (0.03 μg/ml, 0.06 μg/ml, and 0.06 μg/ml) [74]. These isolates were recovered from patients with bloodstream infections treated with caspofungin. Echinocandin MICs for the initial isolates were low (WT by *FKS* sequence), but they increased following therapy [74] (data not shown in Table 4).

IDSA Recommendations for Antifungal Susceptibility Testing

For *Candida* Infections Due to recent trends, the IDSA recommends the determination of azoles and echinocandin MICs for: (i) *C. glabrata* isolates, (ii) isolates from blood/sterile sites, and (iii) isolates recovered from treatment failures, breakthrough infection, or limited therapeutic options (e.g., adverse events, allergies, or previous exposures) [7••].

For *Aspergillus* Infections Again, the recommendation is to test *Aspergillus* isolates from patients failing therapy or for epidemiological purposes [8••]. Ideally, isolates recovered prior to and during therapeutic failure should be tested together.

For Non-*Candida* and Non-*Aspergillus* Species In addition to isolates from sterile/deep sites, the recommendation is to test isolates from patients previously exposed to an antifungal agent, rare/emerging, or species known to be less susceptible to antifungal agents.

Conclusions

Available standardized antifungal susceptibility testing methods are internationally used. CLSI/EUCAST triazole and echinocandin BPs and ECVs/ECOFFS have been established for some *Candida* spp. and *Aspergillus* spp. Reference BPs or ECVs could be useful MIC cutoffs as predictors of failure/success to therapy (BPs) or to identify mutant/non-WT isolates (ECVs). However, other factors can influence therapeutic outcome in addition to microbiological clearance, because this relationship is dependent on the combination of the MIC and the factors discussed above. Current knowledge of genetic resistance mechanisms among *Candida* (triazoles and echinocandins) and *Aspergillus* spp. (triazoles) is valuable since antifungal resistance continues to spread, including multidrug resistance. However, resistance mechanisms have not been elucidated and BPs are not available for other antifungal agents/species (e.g., less common or emerging species or new agents).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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