Curr Fungal Infect Rep
DOI 10.1007/s12281-017-0282-9

Antifungal Susceptibility Testing of *Candida* and *Cryptococcus* Species and Mechanisms of Resistance: Implications for Clinical Laboratories

Catriona L. Halliday[1] · **Monica A. Slavin**[2] · **Sharon C.-A. Chen**[1,3]

Abstract
Purpose of Review Resistance to antifungal drugs amongst *Candida* species is a growing concern, and azole resistance may be emerging in *Cryptococcus* species. This review provides a contemporary perspective, relevant to the clinical mycology laboratory, of antifungal susceptibility testing of these fungi, focussing on the challenges of phenotypic and genotypic methodologies to detect drug resistance.
Recent Findings Standardised CLSI and EUCAST broth microdilution (BMD) susceptibility testing methods are the benchmark to determine clinical breakpoints (CBPs) and/or epidemiological cut-off values (ECVs) MICs for *Candida* and *Cryptococcus* spp. Commercial methods may be used but caution is required when employing BMD CBPs/ECVs to interpret results. Species-specific CBPs/ECVs for *Candida* spp. generally correlate well with predicting likelihood of therapeutic failure or of presence of a drug resistance mechanism with the exception of the echinocandins where the presence of specific *FKS* gene mutations and not the MIC correlates most accurately with clinical outcome. The relationship of presence of one or more mechanisms of azole resistance and drug MICs is uncertain. Next generation sequencing technology is offering insights into the relationships between susceptibility results obtained by phenotypic and genotypic methods. For *Cryptococcus* spp., CBPs are not established but species- and genetic type-specific EVCs are useful for guiding therapy where clinically indicated. Isolates of genotype VGII appear to exhibit the highest MICs.
Summary Antifungal susceptibility testing of yeasts is important to detect drug resistance. For *Candida* spp., MICs have clinical utility for the azoles but detecting echinocandin resistance by genotypic methods is preferred. For *Cryptococcus* spp., ECVs are useful in guiding therapy.

Keywords Antifungal susceptibility · Drug resistance · *Candida* · *Cryptococcus*

Introduction

Invasive fungal disease (IFD) caused by *Candida* and *Cryptococcus* species represents significant morbidity with mortality rates as high as 70–75% [1, 2]. Rapid diagnosis including detection of drug resistance is essential for early initiation of appropriate antifungal treatment [3]. *Candida* comprise the commonest yeasts encountered in the clinical mycology laboratory where *Candida albicans* remains the most frequent; however, the prevalence of species with reduced susceptibility or resistance to antifungal drugs, most notably *Candida glabrata*, is increasing due to selective pressures from increased antifungal drug use [4]. *C. glabrata* and *Candida parapsilosis* are the second and third most common causes of candidaemia in Australia [5], North America [6] and Northern Europe [7•] whilst in India [8] and Latin America [9], *Candida tropicalis* is the more, if not most, frequent species, although in some studies from Latin America, *C. parapsilosis* is the second most common species after

This article is part of the Topical Collection on *Advances in Diagnosis of Invasive Fungal Infections*

✉ Sharon C.-A. Chen
sharon.chen@health.nsw.gov.au

[1] Centre for Infectious Diseases and Microbiology Laboratory Services, ICPMR – NSW Pathology, Westmead Hospital, 3rd Level, ICPMR Building, Darcy Road, Sydney, NSW 2145, Australia

[2] Deparment of Infectious Diseases, Peter MacCallum Cancer Centre, Melbourne, Australia

[3] Marie Bashir Institute for Infectious Diseases and Biosecurity, The University of Sydney, Sydney, Australia

Published online: 08 July 2017

C. albicans. Of concern, the frequency of azole resistance and of echinocandin resistance within *C. glabrata* has reached up to 30 and 23%, respectively, in some countries, and may be centre-specific. Currently, rates of azole resistance amongst *C. tropicalis* are estimated at 8–16.7% [5, 8].

Other than *Candida*, the two main species of the *Cryptococcus neoformans-Cryptococcus gattii* complex (*C. neoformans* and *C. gattii*) are typically the next most common yeasts encountered. Each of *C. neoformans* and *C. gattii*, incorporating their hybrid species, comprises four major molecular types (VNI-VNIV and VGI-VGIV, respectively) [10]. As these molecular types may be associated with differences in virulence and antifungal susceptibility [11•, 12], elucidating their identification and drug susceptibility profiles is important. *C. gattii* isolates are reported to be less susceptible to some antifungal drugs than isolates of *C. neoformans*, and in some studies, there have been statistically significant differences in fluconazole minimum inhibitory concentration (MIC) values amongst molecular types of *C. gattii*, with type VGII having the highest fluconazole geometric mean (GM) MICs [13–15].

This article summarises the methods used in diagnostic mycology laboratories to detect drug resistance and the challenges thereof in *Candida* and *Cryptococcus* species. We also briefly describe the major molecular mechanisms of drug resistance and whether phenotypic susceptibility testing methods correlate with the molecular basis for resistance. For this review, the term '*Candida*' is used to encompass the main ascomycetous yeasts, which have historically been pooled into the artificial genus *Candida* (including *Pichia*, *Clavispora* and *Meyerozyma*) [3, 4].

Antifungal Susceptibility Testing Methods

In vitro antifungal susceptibility testing is performed to guide selection of antifungal drug therapy but is also important to detect drug resistance, or probability that treatment will fail [16]. Although species identification can help predict resistance in intrinsically resistant species, e.g. *Candida krusei* and fluconazole, it cannot infer presence of acquired resistance.

There are two reference standards for susceptibility testing of yeasts: the Clinical and Laboratory Standards Institute (CLSI) methods [17] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods [18]. Both are based on broth microdilution (BMD) and, despite technical differences, e.g. inoculum size and determination of MIC endpoint, are able to reliably discriminate between susceptible 'wild-type' strains (no acquired resistance mechanism) and resistant strains with intrinsic or acquired resistance [16]. Both methodologies have developed clinical breakpoints (CBPs) [19] and epidemiological cut-off values (ECVs) for *Candida* spp. [20]. The CBP is the concentration (mg/L) of drug which defines whether the organism is susceptible, susceptible-dose dependent (S-DD) or resistant and is based on the in vitro response of the organism to the antifungal at levels corresponding to blood or tissue levels attainable with usually prescribed doses of that agent [17]. The ECV is the upper limit of the wild-type population and usually incorporates 90–95% of a given population for a specific agent [21•], i.e. it is the MIC concentration that separates a population into those with and without acquired and/or mutational resistance based on their phenotype [22]. CBPs and/or ECVs have been established for the more common species of *Candida* for most antifungal drugs [7•, 18–20, 23] and ECVs also developed for *Cryptococcus* species (see section on '*Cryptococcus*'). Importantly, these CBPs or ECVs are species-specific and the reader is referred to the appropriate CLSI or EUCAST documents.

Because these 'gold standard' reference techniques require 48–72 h to perform [24], many clinical laboratories utilise commercial tests such as the Sensititre® YeastOne® (TREK Diagnostics Systems, Cleveland, Ohio) and Etest (bioMérieux, Marcy d'Etoile, France) for susceptibility testing for convenience, but standardisation of these methods against the reference BMD tests is not absolute for all drug-bug combinations and may result in the misclassification of both 'susceptible' and 'resistant' *Candida* strains when reference CBPs are applied [25]. Guinea et al. [26] have demonstrated that the Etest performed directly on positive blood samples performed well with the azoles (fluconazole, voriconazole and isavuconazole) compared with the CLSI standard [17], offering a rapid (24 h) susceptibility result in patients with candidaemia. The Sensititre® YeastOne® has also been used to test susceptibility of cryptococcal isolates (see section on '*Cryptococcus*').

The Vitek 2 (bioMérieux) automated yeast identification and susceptibility system, which reduces result variability by using spectrophotometric reading of results [27], provides MIC results more rapidly within 14–18 h [28]. Comparative studies between CLSI BMD [17, 19] and Vitek 2 MICs in *Candida* species have generally shown excellent essential agreement (EA) (within 2 dilutions) as well as categorical agreements (CA) for fluconazole (97.9 and 96.8%), voriconazole (96.7 and 96.5%), caspofungin (99.5 and 99.8%), micafungin (98.6 and 98.2%) and posaconazole (95.6 and 98.1%) [27, 29]. However, intra-species discrepancies in MICs were observed particularly for *C. glabrata* and *C. krusei*; the Vitek 2 is also unable to detect the susceptibility of *C. neoformans* isolates to voriconazole [30]. Further, when *Candida auris* isolates were tested for amphotericin B susceptibility, highly elevated MICs were noted but with only 10% EA between Vitek and the CLSI reference method which may lead to the selection of inappropriate therapy [31].

Candida Species

Azole Resistance in *Candida*

The widespread use of triazoles has resulted in both primary resistance, observed as a shift towards inherently less susceptible species (e.g. *C. glabrata* and *C. krusei*), and secondary resistance, which selects for resistant sub-populations in normally susceptible strains (e.g. *C. albicans*) [32]. Secondary or acquired resistance is less predictable and may be underreported [7•]. The prevalence of azole resistance varies with species and even within species. *C. glabrata* exhibits intrinsic decreased susceptibility to azoles and may develop high-level resistance following azole exposure [33•]. Amongst *C. albicans*, bloodstream isolates have been associated with the lowest incidence (0–5%) of azole resistance [34, 35] with those from the oropharynx after fluconazole treatment exhibiting higher resistance rates [33•]. Surveys of invasive candidiasis have noted overall fluconazole resistance rates of 10–25% with many isolates, particularly *C. glabrata*, cross-resistant to other triazoles [24, 33•]. Isavuconazole, the most recent marketed azole, has broad in vitro activity against *Candida* species similar to that of voriconazole; up to 15% of *C. glabrata* and *C tropicalis* may have non-WT MICs depending on the centre [36•].

Azole resistance implicating a range of candidate genes inked to resistance has been most extensively studied in *C. albicans*. Studies have examined orthologous genes in *C. parapsilosis* and the recently described *Candida auris* with mixed results whilst comparatively little is known about azole resistance in *C. tropicalis* [33•, 37•]. There are four main mechanisms of azole resistance in *Candida* species (summarised in Table 1): (i) induction of drug efflux pumps encoded by the Multi-Drug Resistance (*MDR*) and *Candida* Drug Resistance (*CDR*) genes, which decrease drug concentrations at the site of the target enzyme, 14-α-sterol demethylase; (ii) acquisition of point mutations in the *ERG11* gene which encodes the target enzyme resulting in a target with reduced affinity for azoles; (iii) overexpression or up-regulation of *ERG11* which helps overcome azole activity and (iv) development of bypass pathways, linked to mutations of the *ERG3* gene, which negate the fungal membrane-disruptive effects of azoles [16]. A resistant strain may have more than one mechanism active with additive effects. Induction of *CDR* gene-encoded efflux pumps usually affects all azole drugs and is sufficient for resistance. However, efflux pumps encoded by *MDR* genes are usually selective for fluconazole resistance only [16].

Detecting Azole Resistance in *Candida*

In vitro susceptibility testing is useful to guide classification of an isolate as susceptible or resistant, although it cannot determine if an isolate harbours a mechanism of acquired antifungal resistance [38]. Nonetheless, for the azoles, with the exceptions of isavuconazole where data is relatively lacking and *C. glabrata*, species-specific interpretative CBPs have in general been shown to have utility in predicting outcomes [39]. Hence, for the clinical laboratory, phenotypic MIC testing remains a core function.

Conversely, molecular assays have the ability to determine the underlying genetic basis of triazole resistance [32]. However, they have the drawback in that they are not standardised and that in many circumstances, correlation of presence of a genetic change with phenotypic resistance is uncertain (Table 1) [24].

Mutations in the *ERG11* gene are clustered into three 'regional hotspots' within the genome corresponding to amino acid positions 105 to 165, 266 to 287 and 405 to 488 [40]. Direct sequence analysis of the *ERG11* gene is the most direct method of detecting these point mutations [41] but other methods include a simple and rapid (2 h) padlock probe and rolling circle amplification (RCA)-based method for their detection [42] with good specificity. Whether such gene mutations confer phenotypic resistance to the azoles in diploid *Candida* species such as *C. albicans* is complicated by the need to demonstrate mutations in one or more alleles, and to demonstrate a causal effect by experiments involving matched pairs of susceptible and resistant isolates from the same patient or by gene expression studies [43].

Quantitative reverse transcriptase-PCR (RT-PCR) has now replaced semi-quantitative Northern blot analysis to assess transcript levels of *CDR1*, *CDR2* and *MDR1* genes [44, 45]. Unlike specific mutation detection, expression profiling of

Table 1 Genetic mechanisms leading to resistance by antifungal compound in *Candida* species and molecular detection tools (modified from Perlin [32] and Cuence-Estrella [24])

Antifungal agent	Genetic target	Availability of reliable molecular tools	Correlation measured MIC with presence of gene mutation	Integration into routine daily use
Amphotericin B	No	No	No	No
Azoles	*MDR1*	Yes	Uncertain	No
	CDR1	Yes	Uncertain	No
	CDR2	Yes	Uncertain	No
	ERG11	Yes	Uncertain	No
	ERG3	Yes	Uncertain	No
	TAC1	Yes	Uncertain	No
Echinocandins	*FKS1*	Yes	Yes	Strongly considered
	FKS2	Yes	Yes	Strongly considered
	FKS 3	Yes	Yes	Strongly considered

these genes requires cell cultures grown in the presence/absence of drug and threshold levels need to be associated with the resistance phenotype [32]. One study used RT-PCR to quantify mRNA levels in *CDR1*, *CDR2*, *ERG11* and *MDR1* genes in the absence of azole exposure in *C. albicans* isolates with variable fluconazole susceptibilities [44]. *ERG11* expression levels were significantly higher in the fluconazole resistant isolates compared with S-DD and susceptible isolates, as well as in S-DD isolates compared with the susceptible isolates. Additionally, *CDR1* and *CDR2* overexpression, but not *MDR1*, was noted amongst resistant but not susceptible isolates. The assay had improved sensitivity, specificity and speed of analysis over Northern hybridization and was able to quantify very low and high mRNA levels [44].

A recent study by Xisto et al. [9] found 50% of *C. tropicalis* isolates ($n = 22$) were phenotypically resistant to one or more azoles and six were pan-azole resistant. By conventional PCR and DNA sequencing to amplify hot spot region 1 (HS1) (amino acid positions 641–649) and hot spot region 2 (HS2) (amino acid positions 1357–1364) in *ERG11*, a novel amino acid substitution at location K143R, near the active binding site of *ERG11*, together with an up-regulation of the ABC transporters, was associated with phenotypically pan-azole resistant isolates. K143R has been associated with fluconazole resistance in *C. albicans* [9].

Most recently, next generation sequencing (NGS) has been used to analyse genes commonly linked to azole resistance (*ERG11*, *ERG3*, *TAC1* and *CgPDR1*) in *C. albicans*, *C. glabrata* and *C. parapsilosis* [46]. NGS provides a genome-wide view of gene mutations and is able to detect new combinations of mutations that would probably be missed by targeted DNA sequencing [46]. Lockhart et al. [37•] compared *ERG11* amino acid sequences for *C. auris* obtained by NGS to *C. albicans ERG11* and identified nine amino acid substitutions associated with azole resistance as well as three additional hotspot amino acid substitutions (F126T, Y132F and K143R) that are either proposed or proved to confer fluconazole resistance in *C. albicans*. However, NGS is less accessible to clinical laboratories than Sanger sequencing and although costly (AUD 80/sample), these costs are likely to decrease with technological advancements.

Mechanisms of Echinocandin Resistance in *Candida*

Echinocandin resistance in *Candida* species (including *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. dubliniensis*) has been linked to point mutations in two 'hotspot' regions (HS1: region 640–650 and HS2: 1345–1365) of the *FKS1* gene, which encodes the major subunit of 1,3-β-D-glucan synthase complex. Additional mutations in the *FKS2* gene of *C. glabrata* are also linked to resistance [3, 16, 47, 48]. The most frequent amino acid changes in *C. albicans* is at positions Ser641 and Ser645, whereas in *C. glabrata*, amino acid substitutions at positions Ser629 in *FKS1* and Ser663 and Phe659 in *FKS2* are the most prominent [49]. These unique mutations result not only in higher MICs to one or more echinocandins but also clinical failure [50]. In *C. glabrata*, the presence of *FKS* mutations are an independent risk for treatment failure in patients with invasive candidiasis [51•].

For most *Candida* species, echinocandin resistance remains relatively low, for example, at <3% in *C. albicans* [52]; however for *C. glabrata*, echinocandin resistance rates is rising (from 4.9 to 12.3% in 10 years in one US hospital) [50], and many isolates also show co-resistance to azoles [49, 53, 54]. Naturally occurring polymorphisms in the *FKS* genes (P660A at the 3′-end of HS1) [21•] of the *C. parapsilosis* complex (*C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis*) and *C. guilliermondii* result in higher echinocandin MICs, relative to other *Candida* spp., but these have not been associated with treatment failure [49].

Detecting Echinocandin Resistance in *Candida* Species

Both CLSI [55] and EUCAST [7•] have established species-specific CBPs for the echinocandins, although EUCAST has not set CBPs for caspofungin. In addition, ECVs, which define the upper limit of the wild-type MIC population in the absence of a known *FKS* resistance mechanism, have been defined for anidulafungin and micafungin against common *Candida* species [20].

For the echinocandins, in vitro MIC susceptibility testing is not able to completely distinguish WT isolates from echinocandin-resistant (*FKS* mutant) isolates, especially for caspofungin [7•] where there is substantial inter-laboratory variability of results even with reference methodologies; this has led to isolates falsely classed as 'resistant'. This limitation of MIC testing may be overcome by molecular testing to detect *FKS* mutations by either DNA sequencing, real-time PCR or NGS (Table 1) [46, 48, 56, 57]. Molecular testing is preferred because (i) an *FKS* mutation is a primary clinical indicator for reduced therapeutic response; (ii) the number of *Candida* strains exhibiting resistance in vitro is low and (iii) there is a limited spectrum of mutations conferring resistance [24, 51•]. A multiplex PCR assay detected resistance mutations in *C. glabrata*, with 98% concordance between mutation detection and echinocandin MICs [48]. The assay was inexpensive, simple and quick (<4 h), but was unable to detect a three nucleotide mutation encoding the F659 at Fks2p. DNA sequencing is accurate for identifying *FKS* mutations but requires multiple PCR assays, and is costly. Pham et al. [58] developed a rapid, high-throughput Luminex microsphere-based assay to identify *C. glabrata FKS* mutants, but this approach is also expensive.

NGS has also been used to analyse *FKS* gene mutations in clinical isolates of *C. glabrata*, demonstrating high concordance between drug MICs and genomic variation [46, 57]. Biswas et al. [57] recently used NGS to retrospectively study three strain pairs of *C. glabrata* from three patients where antifungal resistance developed during treatment. For two of three isolate pairs, there was a >60-fold increase in MICs to all echinocandins and NGS detected mutations in either the *FKS1* (S629P) or *FKS2* (S663P) genes of the resistant isolate.

Amphotericin B Resistance in *Candida*

Despite more than 50 years of clinical use, resistance to amphotericin B is uncommon in *Candida* species (1–3%), possibly due to its fungicidal activity limiting the selection of mutants [3]. There have been reports of increasing MICs to amphotericin B amongst *C. krusei* and *C. glabrata* isolates and intrinsic polyene resistance is reported for *Candida lusitaniae*, but resistant isolates are difficult to reproduce in vitro [59]. There are no reliable molecular tools to detect amphotericin B resistance (Table 1) and phenotypic susceptibility testing to determine the MIC remains the more clinically relevant [24] despite there being no CBPs for polyenes. Most clinicians use a MIC ≥1.0 μg/mL to flag 'possible drug resistance' [59].

Cryptococcus neoformans

The widespread practice of fluconazole use for long-term suppressive therapy of cryptococcosis, especially in patients with HIV/AIDS, has led to concern for development of fluconazole (and other azole) resistance [60, 61]. The guidelines of the Infectious Diseases Society of America (IDSA), however, consider primary resistance of *C. neoformans* to fluconazole not to be a significant clinical problem [60] and that susceptibility testing be performed as guided by clinical need, e.g. clinical failure, rather than as routine. This recommendation is based on findings of several studies from disparate geographic regions where the prevalence of fluconazole 'resistance' was <5% (based on a MIC of >16 mg/L—see below) [62–64].

Yet emergence of azole resistance or non-susceptibility may be growing. A global study from 1997 to 2007 documented an increase in resistance to fluconazole when results from time periods 1997–2000 (7.3%), 2002–2004 (10.9%) and 2005–2007 (11.7%) were compared, being most evident for isolates from the Asia Pacific, Africa and Latin America [65]. The rate of fluconazole-non-susceptible *C. neoformans* isolates also increased significantly from 2001 to 2012 in Taiwan [66] as in Uganda where between 2010 and 2014, there was an upward shift in fluconazole MICs (MIC_{90} 32 mg/L vs. 8 mg/L in 1998–99) [67]. However, these results are confounded by the use of different methodologies across studies and different criteria to define 'resistance' or 'non-susceptibility'. Similar variation is noted for studies reporting different (low) rates of resistance to amphotericin B and 5-flucytosine but employing cut-off MIC values [68].

Clinical Breakpoints and Epidemiological Cut-off Values

Unlike for *Candida* spp., the availability of methodologies to enable recognition of drug-resistant isolates by interpretative CBPs by both CLSI and EUCAST to infer susceptibility/resistance is not well defined. It is also unclear whether the current CBPs for *Candida* spp. are appropriate for *Cryptococcus*; hence, there are no standard breakpoints for *C. neoformans* or *C. gattii* for any antifungal drug. CBPs are based not only on parameters such as MIC distributions, pharmacokinetic (PK) and pharmacodynamic (PD) data but also on clinical outcomes of therapy. In cryptococcosis, the impact of drug MICs on clinical outcomes is uncertain with relatively few systematic data. An earlier review found that by using CLSI M27-A3 BMD methodology [17], high fluconazole MICs (>8–16 mg/L) for *C. neoformans* were predictive of clinical failure [69] as did the findings of Aller et al. [70] where reduced treatment efficacy was noted when the fluconazole MIC was >16 mg/L. However, others report that MICs do not predict clinical outcomes as also noted for amphotericin B and 5-flucytosine [67, 71].

As CBPs are not validated for the azoles, amphotericin B or 5-flucytosine, ECVs (based mostly on MIC distributions) have been proposed to help identify presence of resistance mechanisms that could lead to reduced susceptibility [72]. In a multicentre study, using CLSI M27-A3 methodology [17], ECVs of amphotericin B (0.5 to 1.0 mg/L) and 5-flucytosine (4 to 16 mg/L) were proposed where these values are both species- and molecular type-specific (Table 2). Similarly, the same consortium established ECVs for the four major azoles in clinical use, based on CLSI WT MIC distributions (Table 2): the species- and molecular type-specific ECVs of fluconazole were 8–32 mg/L, of itraconazole, 0.12–0.25 mg/L, and of voriconazole and posaconazole, 0.12–0.25 mg/L and 0.25–0.5 mg/L, respectively [73]. A similar study by the CDC, USA, using the Sensititre® YeastOne® system (TREK Diagnostics) established ECVs for each azole and the molecular types of *C. gattii* [11•]. In general, ECVs were identical or similar or to those of Espinell-Ingroff et al. [72, 73] and are shown as numerical values within brackets in Table 2.

There are a number of important issues around the interpretation of ECVs for *Crypotococcus*. Because clinical outcome data following treatment of cryptococcosis and PK/PD data are relatively lacking for the antifungal drugs used, the ECVs are established as a surrogate for CBPs to define WT MIC distributions and identify isolates that may not respond to therapy. For *Cryptococcus*, it is evident from Table 2 that the development of a single 'one size fits all' ECV for azoles or

Table 2 Epidemiologic cut-off values for the major species of *C. neoformans* and *C. gattii* (modified from references [11•] and [72]) to antifungal drugs

Cryptococcus species and molecular type	Antifungal agent	ECV (mg/L)
Cryptococcus neoformans		
VNI	AMB	1
Non-typed		0.5
VNI	5-FC	8
Non-typed		16
VNI	FLU	8
VNIII		16
Non-typed		16
VNI	ITC	0.25
Non-typed		0.5
VNI	VRC	0.25
VNIII		0.25
VNIV		0.12
Non-typed		0.25
VNI	POS	0.25
Non-typed		0.25
Cryptococcus gattii		
VGI	AMB	0.5
VGII		1
VGIIa		0.5
Non-typed		1
VGI	5-FC	4
VGII		16
VGIIa		16
Non-typed		4
VGI	FLU	8 (8)
VGII		32 (32)
VGIIa		8 (−)
VGIII		8 (8)
VGIV		16 (16)
Non-typed		8 (−)
VGI	ITC	0.5 (0.5)
VGII		0.5 (1)
VGIIa		0.5 (−)
VGIII		0.5 (1)
VGIV		1.0 (1)
Non-typed		0.5 (−)
VGI	VRC	0.5 (0.12)
VGII		0.25 (0.5)
VGIIa		0.25 (−)
VGIII		−(0.12)
VGIV		−(0.25)
Non-typed		0.25 (−)
VGI	POS	0.5 (0.5)
VGII		−(1.0)
VGIII		−(0.12)
VGIV		−(1)
Non-typed		0.5 (−)

AMB amphotericin B, *5-FC* 5-flucytosine, *FLU* fluconazole, *ITC* itraconazole, *POS* posaconazole, *VRC* voriconazole

indeed for other agents that covers all *C. neoformans* and *C. gattii* molecular types is impractical and may be even incorrect.

Nonetheless, all azoles and amphotericin B have good in vitro activity against *Cryptococcus*. For fluconazole, higher GM MICs for *C. gattii* molecular-type VGII may be evident, and for all *Cryptococcus*, the GM MICs of itraconazole, posaconazole and voriconazole are lower than that for fluconazole. Selection of one azole over another purely on the basis of MICs is flawed since there are no CBPs to recommend one over another. Fluconazole (and to lesser extent itraconazole) has been time-tested as the preferred azole for maintenance therapy of cryptococcosis, although the newer azoles also have a therapeutic role as second line or alternate agents where fluconazole is not able to be used [60].

Of note also is that the overall ECV for fluconazole for *Cryptococcus* would be in the range that most *Candida* species would be considered resistant (MIC > 4 mg/L). The same is true for the other azoles. So whilst an ECV of 32 mg/L, for example, may seem high compared with *Candida* ECVs or CBPs, it may be that these values are in the susceptible range for *C. neoformans* and *C. gattii*, especially given the high dose of fluconazole used to treat cyptococcosis [60].

Isavuconazole

Isavuconazole has good activity against *C. neoformans* and *C gattii*. In one study, the GM MIC for 90 *C. gattii* isolates was 0.057 mg/L with no significant increase observed for the molecular type VGII. In absolute terms, isavuconazole had about tenfold greater activity against *C. gattii* compared with fluconazole [74]. Another study focussing on *C. gattii* also determined that isavucoanzole had the lowest MIC_{90} (0.125 mg/L) for all isolates (GM MIC 0.051 mg/L) [15]. Both these studies employed CLSI M27-A3 methodology [17]. Thompson et al. [75] compared the isavuconazole Etest (A Biodisk, Solna, Sweden) with CLSI broth microdilution and found a 97.8% agreement between these methods, without major discrepancies (>2-well dilution difference). The GM MICs for *C. gattii* and *C. neoformans* were 0.03 and 0.024 mg/L, respectively by Etest and 0.027 and 0.023, respectively, by broth microdilution [75]. However, clinical correlation with these results is lacking.

Methods for Susceptibility Testing of *Cryptococcus*

Ideally, the CLSI and EUCAST reference BMD methodology is the preferred method for performing susceptibility testing [17, 18]. The good agreement by Etest for isavuconazole susceptibility testing is noted above [75]. Cuenca-Estrella et al. [28] compared the Vitek 2 Antifungal susceptibility system (AST-YS01; bioMerieux Inc., Hazelwood, MO) with the CLSI and EUCAST reference procedures to determine susceptibility to amphotericin B, 5-flucytosine, fluconazole and voriconazole for 16 *C. neoformans* isolates; the average EA was 92%, and the Vitek 2 system (bioMerieux) provided

results more rapidly and was an easier alternative for clinical laboratories.

Mechanisms of Antifungal Drug Resistance

C. neoformans strains that manifest resistance to every class of antifungal drug have been described [69]. For 5-flucytosine, resistance occurs by mutations within the pyrimidine pathway specifically linked to a single mutation within the *FCY1* or *FYC2* gene [76]. Whether their resistance can be predicted by non-WT MIC values for 5-flucytosine in a given population is uncertain. However, the clinical utility of 5-flucytosine lies in its synergistic activity with amphotericin B so despite drug resistance, it may still be useful [60]. Resistance to amphotericin B may occur through defects in the target enzyme sterol delta 8→7 isomerase, other mutations in sterol synthesis genes or an increase in drug efflux [77]. Amphotericin B MICs of >2 mg/L have been associated with presence of more than one of these defects with clinical failure.

The molecular basis of azole resistance in *C. neoformans* is also not well studied. One resistance mechanism is through the duplication of chromosome 1 and consequently of two of its resident genes—*ERG11* and *AFR1* which encodes for an ABC transporter [78, 79] where up-regulation of *AFR1* is involved in resistance to fluconazole. Unlike *Candida* species, however, only a limited number of *ERG11* mutations have been reported to confer azole resistance; these include those leading to the amino acid substitutions G484S and Y145F [80, 81]. That leading to G484S was present in isolates with fluconazole MICs of 16–32 mg/L whilst the substitution Y145F conferred resistance to both fluconazole (MIC >256 mg/L) and voriconazole (≥2 mg/L), although interestingly increased susceptibility to posacoanzole and itraconazole. Of note, Gast et al. [82] examined the roles in azole resistance of *ERG11* overexpression or mutations in the *ERG11* gene in 25 *C. gattii* isolates with high MICs (GM MIC 20 mg/L) and found no correlation between either molecular-based results [82].

For all drug classes, much remains to be examined regarding the relationship between MICs and resistance mechanisms, in particular in determining the value of current ECVs for different bug-drug combinations in identifying isolates which harbour resistance mechanisms**.** For the present, MIC determination remains important for monitoring trends in susceptibility.

Conclusions

Determination of susceptibility or resistance to antifungal drugs remains important for *Candida* and *Cryptococcus* spp. In vitro MIC testing, particularly for *Candida* spp., is well standardised by CLSI and EUCAST methodologies. Overall, species-specific CBPs and ECVs for *Candida* spp.-, and species-, as well as molecular type-specific ECVs for *Cryptococcus* spp. are helpful in identifying isolates that are more/less likely to respond to an antifungal or are more/less likely to harbour a mechanism of drug resistance. However, for *Candida* spp. and the echinocandins, detection of *FKS* gene mutations linked to resistance, rather than MIC determination, predicts clinical outcomes. In the future, testing for molecular markers of resistance including the use of NGS technologies may become the preferred method. Further work on correlating MICs and ECVs with clinical outcomes and with underlying drug resistance mechanisms is required.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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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