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The impact of new epidemiological cutoff values on *Candida glabrata* resistance rates and concordance between testing methods

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ABSTRACT

Interpretive criteria for *Candida* susceptibility testing were recently revised with the establishment of species-specific epidemiological cutoff values (ECV). To assess the effect of modified cutoff values on *Candida glabrata* resistance rates and agreement between testing methods, we tested the susceptibility of 598 clinical isolates to fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, and amphotericin B using CLSI M27-A3 and E-test methods. The caspofungin MICs clustered above the ECV and below the CLSI cutoff (MIC_{50}, 0.5 μg/mL). Applying the ECV reduced the proportion of itraconazole-nonsusceptible strains from 83% to 0.3% but minimally affected resistance rates of other drugs. Categorical agreement between broth microdilution and E-test was increased for itraconazole and reduced for voriconazole and caspofungin. The current caspofungin ECV may not reproducibly differentiate resistant and susceptible *C. glabrata* strains in hospitals with varying MIC distributions.

1. Background

Candida glabrata has emerged as the second most common cause of invasive candidiasis in many hospitals (Lockhart et al., 2012; Pfaller et al., 2012). The rising incidence of *C. glabrata* infection, especially in older patients, is significant given the inherently reduced susceptibility of this species to azoles and the relatively high frequency with which isolates acquire azole resistance during treatment (Bennett et al., 2004; Sanglard et al., 2001). Echinocandins are preferred as primary treatment of *C. glabrata* infections (Cornely et al., 2012; Pappas et al., 2009), but resistance to this class of drugs is also increasingly encountered (Alexander et al., 2013). Importantly, elevated MICs of azoles and echinocandins are associated with increased rates of treatment failure with these agents (Alexander et al., 2013; Shields et al., 2013). Thus, accurate and reproducible determination of *C. glabrata* drug susceptibility is crucial for clinical decision making and resistance surveillance (Cuenca-Estrella et al., 2012).

The Clinical Laboratory Standards Institute (CLSI) recently proposed a revision of the interpretive criteria for *Candida* spp. susceptibility testing for echinocandins and azoles outlined in document M27-A3 (CLSI, 2008, Pfaller et al., 2011a, Pfaller et al., 2010a, Pfaller et al., 2010b, Pfaller et al., 2011b, Pfaller and Diekema, 2012, Pfaller et al., 2011c). The aim of the revision was to define species-specific clinical breakpoints, i.e., MIC cutoffs that predict clinical response to treatment. For *C. glabrata*, currently available data were insufficient to define clinical breakpoints, and MIC cutoffs aimed at capturing isolates harboring drug-resistance mutations (epidemiological cutoff values [ECVs]) were used as provisionary endpoints. It remains unclear however, whether these new cutoffs allow robust categorization of drug-resistant mutants across different laboratories (Alexander et al., 2013; Ostrosky-Zeichner et al., 2003; Pfaller et al., 2011c; Shields et al., 2013). A second focus of the CLSI efforts was the validation of 24-h MIC measurement for azoles, as a means of more rapid and efficient determination of MICs while eliminating the potential confounding effects of trailing growth (Arthington-Skaggs et al., 2002; Pfaller and Diekema, 2012).

Broth microdilution (BMD) is the standard reference method for yeast susceptibility testing (CLSI, 2008; EUCAST, 2008). However, agar-based methods, such as the E-test (bioMerieux, Marcy l'Etoile, France), are significantly less labor intensive and are widely used in many clinical microbiology laboratories. Generally, high rates of categorical agreement (CA) were found between E-test and BMD methods for *Candida* spp., including *C. glabrata* (Diekema et al., 2007; Pfaller et al., 2003). However, the concordance between these methods using the new ECVs is unknown.

Our aim in the present study was to study a large set of *C. glabrata* clinical isolates and to determine rates of resistance to antifungal drugs using ECV and CLSI cutoffs, the impact of trailing growth on resistance rates, and concordance between E-test and BMD methods.

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2. Materials and methods

2.1. C. glabrata isolates

Candida isolates obtained from both sterile and non-sterile sites from 1 January 2005 through 31 December 2007 were analyzed. All *Candida* isolates were identified to the species level using growth characteristics on CHROMagar *Candida* (CHROMagar, Paris, France) and using the Vitek 2 system with the YST ID card (bioMerieux). Isolates identified as *C. glabrata* were selected for antifungal susceptibility testing. Isolates were suspended in sterile water and stored at −80 °C until use. Prior to testing, each isolate was passaged on potato dextrose agar (HyLabs, Rehovot, Israel) and CHROMagar *Candida* to ensure purity and viability.

2.2. Microbiological testing

Antifungal susceptibility testing for fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin was performed in parallel by BMD in accordance with CLSI document M27-A3 (CLSI, 2008) and E-test (bioMerieux), as detailed below. Amphotericin B susceptibility was determined with E-test.

2.2.1. Broth microdilution

Standard antifungal powders of fluconazole (Pfizer), itraconazole (Janssen), voriconazole (Pfizer), posaconazole (Schering-Plough), and caspofungin (Merck) were obtained from their respective manufacturers. Stock solutions were prepared in water (caspofungin and fluconazole), dimethyl sulfoxide (itraconazole and voriconazole), or polyethyleneglycol (posaconazole). Serial 2-fold dilutions of each antifungal agent were prepared. *Candida* spp. were cultured in RPMI 1640 medium (Sigma, Rehovot, Israel) buffered to pH 7.0 with MOPS (Sigma). 0.1 mL of yeast suspension containing $(1.5 \pm 1.0) \times 10^3$ cells/mL was added to each well of the microdilution trays. The trays were incubated in air at 35 °C, and MIC endpoints were read after 24 h for caspofungin and after 24 h and 48 h for all other agents. The MIC was defined as the lowest concentration to achieve a prominent (~50%) decrease in turbidity, compared with that of a drug-free control. Trailing growth was defined as an increase in the 48 h MIC of at least 2 $\log_2$ dilutions as compared with the 24 h reading. Quality control was assured using the CLSI recommended strains, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 (CLSI, 2008).

E-test was performed according to the manufacturer's instructions (bioMerieux, 2007). In brief, several colonies from 24 h or 48 h pure culture on Sabouraud's agar were homogenized in sterile saline to achieve a turbidity of 0.5 McFarland standard. This inoculum suspension was streaked twice on RPMI agar plates (with L-glutamine and phenol red without bicarbonate, MOPS 0.165 mol/L, 2% glucose, 1.5% Bacto agar, pH 7.0 ± 0.1) to achieve an even lawn, and the plates were allowed to dry completely. E-test strips were applied to the dry agar surface, and plates were incubated at 35 °C. MIC was read at 24 h or after 48 h if insufficient growth was present after 24 h. The MIC was determined as the point at which the inhibition ellipse intersects the strip. For the azoles and caspofungin, significant inhibition of growth (80%) was used to determine the MIC. For amphotericin B, a cutoff of 100% inhibition was used.

We analyzed susceptibility rates using 2 sets of interpretive criteria: the M27-A3 criteria (CLSI, 2008) and the proposed ECV-based criteria (Pfaller et al., 2011a; Pfaller et al., 2010a; Pfaller et al., 2010b; Pfaller et al., 2011b; Pfaller et al., 2011c). A summary of the different cutoff values is provided in Table 1.

Concordance between BMD and E-test was expressed as CA and essential agreement (EA) rates. CA was identified when isolates were categorized similarly as susceptible versus nonsusceptible by both methods. EA was identified when the discrepancy between the 2 methods was no greater than 2 $\log_2$ dilutions.

Table 1
CLSI interpretive criteria and epidemiological cutoff values for *C. glabrata* drug susceptibility testing.

Drug	CLSI M27-A3			ECV (Pfaller et al., 2011c)	
	S	S-DD	R	S	R
Fluconazole	≤8	16-32	≥64	≤32	>32
Itraconazole	≤0.125	0.25-0.5	≥1	≤2	>2
Voriconazole	≤1	2	≥4	≤0.5	>0.5
Posaconazole				≤2	>2
Caspofungin	≤2	NA	>2	≤0.12	>0.12
Amphotericin B				≤2	>2

The CLSI has not defined interpretive cutoffs for amphotericin B because the M27 method does not consistently detect amphotericin B–resistant *Candida* isolates (CLSI, 2008). In general, isolates with amphotericin B MIC >2 μg/mL are likely to be resistant to this drug. We therefore used the E-test method and a cutoff of >2 μg/mL for amphotericin B susceptibility testing.
NA = not applicable.

3. Results

A total of 598 patient-specific *C. glabrata* isolates were included in this study. Isolates were recovered from urine (415 isolates, 69.3%), respiratory tract (74 isolates, 12.3%), vagina (27 isolates, 4.5%), blood (17 isolates, 2.8%), and other clinical specimens (65 isolates, 10.8%).

3.1. Drug susceptibility classification according to CLSI and ECVs

3.1.1. Fluconazole

The proposed fluconazole ECV (32 μg/mL) is similar to the CLSI cutoff for resistance (Table 1 and Fig. 1A). Ten isolates (1.6%) were resistant by CLSI and ECV breakpoints, and 10 isolates (1.6%) were susceptible dose dependent (S-DD) by CLSI criteria (Table 2). The fluconazole trailing phenotype was observed in 230 isolates (38.4%). Comparing resistance rates at 24 h versus 48 h, trailing caused 10 susceptible isolates (1.6%) to be reclassified as S-DD and 9 susceptible or S-DD isolates (1.5%) to be reclassified as resistant (Fig. 1A).

3.1.2. Itraconazole

The ECV for itraconazole (>2 μg/mL) is 4-fold higher than the CLSI resistance cutoff (≥1 μg/mL) (Table 1). According to CLSI interpretive criteria, 24 isolates (4.0%) were itraconazole resistant, and 475 (79.4%) were itraconazole S-DD. Using the ECV, only 2 isolates (0.3%) were itraconazole resistant (Table 2). The trailing phenotype was observed in 398 isolates (66.5%). Analysis of the effect of trailing on 48 h versus 24 h susceptibility rates using CLSI criteria showed that 376 of 478 isolates (78.6%) shifted from the susceptible category to the S-DD category, and 22 isolates (3.6%) shifted from susceptible and S-DD categories to the resistant category (Fig. 1B). MIC determination at 24 h rather than at 48 h lowered the proportion of CLSI S-DD isolates from 79.4% to 19.4% (Fig. 1B). In contrast, the trailing phenomenon did not affect the ECV resistance rate.

3.1.3. Voriconazole

The voriconazole ECV (>0.5 μg/mL) is 4-fold lower than the CLSI resistance cutoff (≥4 μg/mL) (Table 1). Using the ECV, the rate of resistance to voriconazole increased from 0.17% (1 isolate) to 2.6% (16 isolates). Voriconazole trailing was observed in 329 isolates (55.0%). Trailing caused 10 isolates susceptible to voriconazole at 24 h (1.7%) to be reclassified to the S-DD or resistant categories at 48 h according to CLSI interpretive criteria (Fig. 1C). Using the ECV criteria, trailing caused 15 voriconazole susceptible isolates (2.5%) to be reclassified as voriconazole resistant (Fig. 1C).

3.1.4. Posaconazole

No CLSI interpretive criteria have been defined for posaconazole; the proposed ECV resistance criterion for this drug is >2 μg/mL