AUSTRALIAN ANTIFUNGAL SUSCEPTIBILITY DATA 2008 - 2011

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Part 1: The Yeasts

In this article, an update of recent changes to the CLSI antifungal standards for susceptibility testing of yeasts is presented. We also summarise current Australian antifungal susceptibility data for *Candida* and *Cryptococcus*.

A. CLSI M27-A3 and M27-S3 broth dilution standard for yeasts[1,2]

The M27-A3 (most current) standard and M27-S3 informational supplement is intended for testing yeasts including *Candida* species and *Cryptococcus* species (*C. neoformans* and *C. gattii*) by using either a macro or micro broth dilution test system.

1. The M27-A3 standard recommends the use of RPMI-1640 medium supplemented with 0.2% glucose and buffered to a pH of 7.0 with 0.165 mol/L MOPS. However, at the SA Pathology Mycology Unit, we routinely increase the glucose concentration to 20 g/L as documented in the M27-S3 supplement, which allows this modification to simplify endpoint determination.[2] This modification enhances the growth of *Cryptococcus* species and other yeasts that do not readily grow on standard RPMI medium. This modified RPMI glucose broth is now available from Oxoid Australia (TM 4040) in pre-packed 11 ml aliquots ready to use with the Sensititre YeastOne microbroth dilution plates.

2. The M27-A3 standard further recommends the application of less stringent MIC end points for the azoles and the echinocandins (50% inhibition) to improve interlaboratory agreement and also discriminate between putatively susceptible and resistant isolates.[2] Endpoint readings for amphotericin B remains at 100% inhibition.

3. Interpretation of results: For the most part, amphotericin B MICs for *Candida* species cluster between 0.25 and 1.0 µg/ml. However it must be stressed that the M27-A3 standard does not consistently permit detection of resistant strains and isolates with MICs of >1.0 µg/ml should be considered to be likely resistant. No MIC breakpoints for amphotericin B are recognized by the CLSI. However, interpretative breakpoints for *Candida* against fluconazole, itraconazole, voriconazole, 5-fluorocytosine and the echinocandins have been established (Table 1).[2,3] These breakpoints have also been used for isolates of *Cryptococcus* where there is some correlation between elevated MIC and treatment failure.[4] However the following caveats for fluconazole should now be noted[2]: (1) The 8 µg/ml susceptibility cutoff for fluconazole is not known with certainty. (2) The data would permit selection of either 4 or 8 µg/ml as the cutoff. (3) All *C. glabrata* isolates with an MIC ≤32 µg/ml should be reported as SDD. All *C. krusei* isolates should be reported as resistant (regardless of the MIC result).[2]

Table 1. Antifungal Breakpoints for Candida.[2,3]

Agent	S	SDD or I	R
Fluconazole	≤ 8.0 mg/ml.	16-32 mg/ml.	≥ 64 mg/ml.
Itraconazole	≤ 0.125 mg/ml.	0.25-0.5 mg/ml.	≥ 1 mg/ml.
Voriconazole	≤ 1 mg/ml	2 mg/ml.	≥ 4 mg/ml
*Posaconazole**	no break points set < 1 mg/ml likely to be susceptible		
Echinocandins	≤ 2 mg/ml	> 2 mg/ml not susceptible	
5-Fluorocytosine	≤ 4 mg/ml.	8-16 mg/ml.	≥ 32 mg/ml.
*Amphotericin B**	≤ 1 mg/ml	2 mg/ml.	≥ 4 mg/ml

*Tests for Amphotericin B maybe unreliable on RPMI media; CLSI provides no break point but <1 µg/ml likely to be susceptible.

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4. Trailing ends points remain problematic, particularly for fluconazole and the echinocandins. To help resolve this issue the M27-S3 supplement has provided both 24 and 48 hour microdilution MIC ranges for the two QC strains and 11 systemic antifungal agents.[1,2] Ideally plates should be read at 24 hours whenever there is sufficient growth.

Sensititre® YeastOne™ YO10 Test Panel (manufactured by TREK but supplied in Australia by Dutec Diagnostics).

This is a microtitre broth dilution method based on the CLSI standards described above for the testing of *Candida, Cryptococcus* and *Aspergillus* species. Each test consists of a disposable microtitre plate, which contains dried serial dilutions of nine antifungal agents, anidulafungin, caspofungin, micafungin, 5-Fluorocytosine, voriconazole, posaconazole, itraconazole, fluconazole and amphotericin B. The wells also contain Alamar Blue as a colorimetric indicator, which greatly improves the end point readability by a colour change from blue to pink. Results are expressed as an MIC and comparative studies against the CLSI method have shown favorable results.[5,6] Overall, the Sensititre YeastOne is a robust and reproducible test, easy to set up and the end points are usually clearly visible. It has excellent shelf life and the test also works with moulds, especially those that sporulate freely like *Aspergillus*.

Australian antifungal susceptibility data: Candida and Cryptococcus (2008-2010)

Isolates recovered from patients with invasive candidaisis and cryptococcosis were tested for antifungal susceptibility with the CLSI M27-A3 microbroth susceptibility standardsfor yeasts. The MICs at which 90% (MIC_{90}) of the strains that were tested were inhibited, and the range of MICs, for major *Candida* spp. for common antifungal agents are summarised below.

Table 2a: Echinocandin MICs: *Candida spp.* (echinocandins are not active against *Cryptococcus*).

Species	Anidulafungin		Caspofungin		Micafungin	
	Range (µg/ml)	MIC90 (µg/ml)	Range (µg/ml)	MIC90 (µg/ml)	Range (µg/ml)	MIC90 (µg/ml)
C. albicans (200)	0.008-0.125	0.125	0.016-0.5	0.5	0.008-0.125	0.03
C. glabrata (194)	0.016-2	0.25	0.016-8	1.0	0.008-2	0.06
C. parapsilosis (69)	0.06-8	8.0	0.03-8	2.0	0.016-8	8.0
C. tropicalis (22)	0.03-0.25	0.25	0.03-0.5	0.5	0.03-0.25	0.125
C. krusei (21)	0.03-0.25	0.25	0.125-1	1.0	0.06-0.25	0.5
C. dubliniensis (12)	0.016-8	0.125	0.06-8	0.25	0.016-8	0.06
10 other Candida sp (14)	0.03-0.5	0.25	0.03-8	1.0	0.03-1	0.125

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Table 2b. Fluconazole, voriconazole and posaconazole MICs: *Candida* and *Cryptococcus spp.*

Species	Fluconazole		Voriconazole		Posaconazole	
	Range (µg/ml)	MIC90 (µg/ml)	Range (µg/ml)	MIC90 (µg/ml)	Range (µg/ml)	MIC90 (µg/ml)
C. albicans (362)	0.03-16	2.0	0.008-0.5	0.03	0.008-1	0.06
C. glabrata (303)	0.125-256	128	0.016-8	2.0	0.008-8	8.0
C. parapsilosis (137)	0.25-32	8.0	0.008-1	0.25	0.008-0.5	0.06
C. tropicalis (67)	0.25-8	2.0	0.008-0.5	0.25	0.008-8	0.5
C. krusei (53)	32-128	64	0.125-1	0.5	0.03-1	0.5
C. neoformans (46)	1-64	8	0.03-2	0.125	0.008-0.25	0.25
C. gattii (23)	1-64	16	0.03-2	0.25	0.016-0.125	0.125

Table 2c. Amphotericin B, 5-Fluorocytosine and itraconazole MICs: *Candida* and *Cryptococcus spp.*

Species	Amphotericin B		5-Fluorocytosine		Itraconazole	
	Range (µg/ml)	MIC90 (µg/ml)	Range (µg/ml)	MIC90 (µg/ml)	Range (µg/ml)	MIC90 (µg/ml)
C. albicans (362)	0.03-1	0.25	0.016-64	0.5	0.008-0.5	0.125
C. glabrata (303)	0.008-1	0.5	0.008-0.125	0.03	0.008-16	16
C. parapsilosis (137)	0.016-1	0.5	0.03-64	0.25	0.03-1	0.25
C. tropicalis (67)	0.03-1	0.5	0.03-2	0.125	0.03-0.5	0.5
C. krusei (53)	0.125-1	1.0	2.0-16	16.0	0.25-1	0.5
C. neoformans (46)	0.03-2	0.5	0.03-64	4	0.03-1	0.25
C. gattii (23)	0.03-2	0.25	0.03-64	4	0.003-1	0.25

All isolates of *C. albicans, C. tropicalis* and *C.krusei* were susceptible to all three echinocandins. Only two isolates each of *C. glabrata* and *C. parapsilosis* had high MIC's (>2 mg/ml) to caspofungin, while 10 isolates of *C. parapsilosis* also had high MIC's to anidulafungin and micafungin. Two isolates of *C. dubliniensis* had high MIC's (>2 mg/ml) to all the echinocandins. These results are similar to those published by other studies that show the very low MICs typical for wild-type strains of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. kefyr* and the higher MICs typical of *C. parapsilosis*, *C. guilliermondii*, and *C. lusitaniae* for the echinocandins.[7,8] All isolates tested were also susceptible to amphotericin B. Resistance to fluconazole was detected in isolates of *C. krusei* and *C. glabrata.* The majority of *C. glabrata* strains and some isolates of *Cryptococcus* were found to be susceptible dose dependent (SDD) to fluconazole (MICs 16-32 µg/ml). Resistance to itraconazole (MIC ≥1 µg/ml) was more widespread and also there were a greater number of isolates in the SDD range (MICs 0.25-0.5 µg/ml). This occurred predominantly in isolates of *C. glabrata, C. parapsilosis and C. krusei* and in some isolates of *Cryptococcus*. Voriconazole resistance (MIC ≥4 µg/ml) was limited

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to a few isolates of *C. glabrata*; all voriconazole resistant *C. glabrata* stains were cross resistant to fluconazole and itraconazole. Suggested likely resistance to posaconazole (MIC's ≥2 µg/ml) occurred in 161/303 (53%) of *C. glabrata* isolates and in 1 isolate of *C. tropicalis*.

These results are in keeping with other studies that show intrinsic resistance to antifungal agents by *Candida* and *Cryptococcus* species is largely predictable based on accurate identification of the organism.[9,10] Resistance to amphotericin B and the echinocandins is uncommon, while resistance to the azoles is largely confined to isolates of *C. glabrata* and C. *krusei*. [9,10]

B. CLSI M44-A2 and M44-S3 disk diffusion standard for yeasts[11,12]

The M44-A2 standard and M44-S3 informational supplement is intended for disk diffusion susceptibility testing of *Candida* species[11,12]. The standard includes zone interpretive criteria for caspofungin, fluconazole and voriconazole. It recommends the use of Mueller-Hinton agar supplemented with 0.2% glucose and 0.5 ug/ml methylene blue dye medium.

Mueller-Hinton agar is readily available and shows acceptable batch-to-batch reproducibility, the glucose provides a suitable growth for most yeasts and the addition of methylene blue enhances the zone edge definition. The pH of the medium needs to be between 7.2 and 7.4 at room temperature after gelling. The inoculum is standardized to 0.5 McFarland using a densitometer and plates should be incubated at 35°C for 24 hours. Some strains where insufficient growth has occurred after 24 hours may need to be read after 48 hours incubation.

Commercially prepared paper disks for fluconazole (25 ug) and voriconazole (1 ug) are available from Oxoid and Becton Dickinson. Neo-Sensitabs® (manufactured by Rosco in Denmark) are available in Australia by Dutec Diagnostics. Neo-Sensitabs are cheap, agar diffusion tests are easy to set up and show potential for the screening of large numbers of isolates for resistance.

Susceptibility data using the CLSI M44-A2 disk method representing *Candida* isolates (2007-2009) recovered from genital sites are shown in Table 3.

Table 3: Australian antifungal susceptibility data for *Candida* isolates from recurrent vulvovaginal candidiasis (2007-2009).

Species		Fluconazole			Clotrimazole			Nystatin		
		S	SDD	R	S	I	R	S	I	R
C. albicans	220 (82%)	217	3	0	220	0	0	219	0	1
C. glabrate	35 (13%)	3	25	7	25	9	1	35	0	0
C. parapsilosis	4 (1.5%)	4	0	0	4	0	0	4	0	0
C. krusei	4 (1.5%)	0	1	3	4	0	0	4	0	0
C. tropicalis	3 (1%)	3	0	0	3	0	0	3	0	0
C. kefyr	2 (0.7%)	2	0	0	2	0	0	2	0	0
C. dubliniensis	1 (0.4%)	1	0	0	1	0	0	1	0	0
C. guilliermondii	1 (0.4%)	1	0	0	1	0	0	1	0	0
	270	241	29	10	260	9	1	270	0	1

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A total of 270 *Candida* isolates from patients with a clinical history of recurrent vulvovaginal candidiasis were tested against fluconazole, clotrimazole and nystatin. *Candida albicans* accounted (82%) and *C. glabrata* (13%) accounted for 95% of the isolates. Resistance rates for all the agents tested were very low; 10 isolates against fluconazole (7 *C. glabtata* and 3 *C. krusei*), 1 *C. glabrata* isolate against clotrimazole, and 1 *C. albicans* against Nystatin). All isolates of *C. albicans* were susceptible to fluconazole and clotrimazole, however as expected 20% of *C. glabrata* isolates were resistant to fluconazole, although all were susceptible to Nystatin. A previous USA study of patients with suspected vulvovaginal candidiasis reported the incidence of *C. albicans* as 70% and that for *C. glabrata* as 19%[14]. These authors also reported a low fluconazole resistance rate of 3.7% for *C. albicans* and a similar expected higher rate of 15.2% for *C. glabrata*. In conclusion, species identification was predictable of the antifungal susceptibility.[9,10,13] *Candida albicans* remains the dominant pathogen in vulvovaginal candidiasis and antifungal drug resistance does not appear to be a factor in determining recurrent infection. Finally, the role of non-albicans *Candida* species in vulvovaginal candidiasis remains uncertain and further studies are warranted.[15]

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