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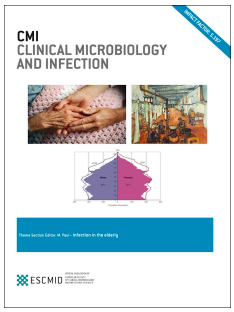

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Original Article

Multiple Colony Antifungal Susceptibility Testing Detects Polyresistance in Clinical *Candida* Cultures: an ECMM Excellence Centers Study

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Abstract

Objectives: Many factors influence the outcome of *in vitro* antifungal susceptibility testing (AFST), including endpoint definition, inoculum sizes, time and temperature of incubation, and growth medium used. This European Confederation of Medical Mycology (ECMM) Excellence center driven study investigated multiple colony testing (MCT) of five separate colonies to investigate the prevalence of polyresistance (PR), defined as heterogeneous MICs from a same-species *Candida* culture irrespective of the underlying resistance mechanism.

Methods: Candida spp. MCT for fluconazole and anidulafungin was performed by Etest© prospectively comprising 405 clinical samples. MCT results were compared to the real-life routine MIC data and PR was assessed. *Candida* colonies displaying strong PR were selected for genotyping using multilocus sequence typing (MLST) and random amplified polymorphic DNA (RAPD) assays for *C. lusitaniae*.

Results: Candida PR was observed in 33 of 405 samples (8.1%), with higher rates for non-*albicans* species (26/186, 14%) than for *C. albicans* (7/219, 3.2%), and for fluconazole than for anidulafungin. MCT detected acquired resistance more often than routine AFST (18/405, 4.5%) and 9 of the 161 investigated blood cultures showed PR (5.6%). MLST and RAPD did not reveal a uniform genetic correlate in strains studied.

Conclusions: This study shows that *Candida* single MIC-values obtained in routine diagnostics may be incidental, as they fail to detect PR and resistant subpopulations reliably. The reasons for PR seem to be manifold and should be regarded as a phenotypical expression of genomic variability

irrespective of the underlying resistance mechanism, which may help to interpret ambiguous and non-reproducible AFST results.

Introduction

Invasive *Candida* infections are an emerging threat with high mortality and morbidity, particularly in immunocompromised patients [1]. Due to an increase of drug resistant species, monitoring and detecting antifungal resistance is important [2]. Multiple colony testing (MCT) for antifungal susceptibility testing (AFST) of *Aspergillus* spp. is recommended [3] as azole-resistant and -susceptible *Aspergillus* strains may be simultaneously present in patient samples [4]. MCT aims to detect resistant subpopulations, which otherwise may lead to breakthrough fungal infections [5,6]. Similarly, MCT for *Candida* samples has been suggested studying its within-host genomic diversity [7]. Next generation-multi locus sequence typing showed that 12% of patient samples contained two unrelated *Candida* strains [8]. In line, longitudinal samples from oral candidiasis showed several genetic and phenotypical changes throughout antifungal treatment, including different co-existing MICs for fluconazole [9].

To ensure the inclusion of intra-sample heterogeneity, EUCAST reference method recommends to pick up five representative colonies for inoculum suspension [10]; by contrast, the more commonly used Etest© method (Gradient strip Test) recommends using ‘a few well-isolated colonies’ [11]. Overall, it is rather coincidental how many colonies are truly used in routine AFST, and improbable that a presumed intra-sample heterogeneity can be displayed by one test per sample.

The aim of this study was to assess the prevalence of intra-sample variance of AFST in a variety of usually sterile specimens and *Candida* species, applying MCT in a prospective multi-center study across Europe. We defined here the term polyresistance (PR) as heterogeneous MICs from a same-species *Candida* culture.

Material and methods

Multiple Colony Testing (MCT)

The frequency of PR within taxonomically identical strains was investigated by separately testing five distinct colonies from each clinical culture. Centers from Austria (2), France (1), and Germany (3) participated, six of them being European Confederation of Medical Mycology (ECMM) Excellence centers (ECMM EC). From March 2020 to March 2021, we prospectively collected 405 clinical samples from sterile regions with growth of *Candida* spp. Mixed yeast infections were separately tested, and no consecutive isolates were used. From each of the original samples, five morphologically identical colonies labeled A-E were separately subcultured on a chromogenic agar for *Candida* (bioMérieux, Marcy l'Etoile, France and BBL, Becton Dickinson GmbH, Heidelberg, Germany) to ensure purity. Species identification was performed for each subculture (A-E) by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) Biotyper (Bruker Daltonik GmbH, Bremen, Germany). AFST with fluconazole and anidulafungin was performed separately for each of the five pure subcultures (A-E), using the Etest© gradient diffusion method according to the manufacturer's instructions [11]. Etest© results were translated to EUCAST breakpoints to categorize the MICs [12]. Categorization for species without EUCAST breakpoints was applied as described elsewhere [13–16].

The results of each test (A-E) were compared to the corresponding other colonies and to the MICs obtained in routine diagnostic assays (Etest© [bioMérieux,], MICRONAUT-AM [MERLIN Diagnostika GmbH, Bornheim, Germany] and Vitek 2 AST-YS 08 card [bioMérieux,]). Differences in categorization and above three log2 dilutions were documented as PR. PR was classified in three categories: Categorically (EUCAST S, I, R) polyresistant MICs above three log2 dilutions (strong PR), categorically (EUCAST S, I, R) polyresistant MICs up to three log2 dilutions (moderate PR), and polyresistant MICs above three log2 dilutions without categorical (EUCAST S, I, R) difference (weak PR). Isolates with PR were separately cryopreserved and collected at Medical University of Innsbruck for further analysis, if indicated.

Reproducibility and Genotyping

For selected polyresistant samples, repetition of AFST by EUCAST microdilution and Etest© was performed [11,12]. Four samples displaying strong PR were selected for genotyping of the five separate isolates. DNA was extracted from fresh culture using Type C bead tubes (Macherey-Nagel, Germany) and the Monarch® genomic DNA purification kit T3010 (New England Biolabs, USA), according to the manufacturer's instructions [17].

Multilocus sequence typing (MLST) was performed for *C. albicans, C. glabrata*, and *C. tropicalis* strains according to the recommendations on https://pubmlst.org [18] with primers published elsewhere (Table S1) [19–21]. The PCR products were sent to Eurofins Genomics (Germany) for Sanger sequencing. Sequencing chromatograms were analyzed in Sequence Scanner (Version 2.0, ThermoFisher scientific) and manually corrected for heterologous sites. MLST profiles were determined using the online tools on https://pubmlst.org [18].

In lack of a MLST scheme for *C. lusitaniae*, we performed RAPD for these strains using three random primers OPA-1, OPA-8, and OPA-10 (Table S2) [22,23]. PCR products were separated in a 1.5% agarose gel at 100 V for 3 h and analyzed with the Gel DocTM EZ Imager and the Image Lab software Version 5.2.1 (both Bio-Rad Laboratories, USA).

Statistical analysis

The sample size was prespecified with 400 samples to have 80% power to detect acquired resistances of at least 3% between MCT and routine AFST as statistically significant using McNemar`s test. McNemar`s test was used to compare detection rates within the same samples between different methods. Pearson`s chi-square test was applied to compare detection rates between samples of different species. P-values <0.05 were considered to indicate statistical significance. Statistical analyses were performed with IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA).

This project was part of a laboratory quality management study within ECMM Excellence Centres; the study was approved by the hospital's ethics committee (Study no. 1166/2018).

Results

MCT for fluconazole and anidulafungin was performed for 405 clinical samples with *Candida* spp. in six centers. Sample origins are summarized in Table 1, MICs from routine diagnostic assays are summarized in Table S3.

Altogether, PR of any category was obtained in 8.1% (33/405) of all *Candida* culture samples (Table 2). Predominantly PR was moderate, meaning categorically (EUCAST S, I, R) polyresistant MICs within three log2 dilutions, with often borderline R MIC values. PR was significantly more

142 frequent in non-*albicans* species than in *C. albicans* (26/186, 14.0% versus 7/219, 3.2%, p < 0.001).
143 There was a tendency towards higher PR rates for fluconazole than for anidulafungin, however,
144 this trend was not statistically significant (22/405, 5.4% versus 13/405, 3.2%, p = 0.08). In *C.*
145 *albicans* isolates with fluconazole MICs above 1 µg/ml, 3/7 showed moderate PR in MCT. In *C.*
146 *glabrata*, 8/11 PR samples showed fluconazole MICs above 8 µg/ml in routine AFST, while
147 reversely, 8/43 samples with fluconazole MICs above 8 µg/ml showed PR upon re-testing. After
148 exclusion of species with clinical breakpoints above 0.064 µg/ml for anidulafungin, 9/21 samples
149 with high MICs above 0.016 µg/ml showed PR in MCT (43%). To estimate the implications of PR,
150 differences between routine results and MCT were labeled as very major error (VME, S vs R),
151 major error (ME, R vs S), minor error (mie, S vs I or I vs R), and very minor error (vmie, I vs S or R
152 vs I). MCT revealed 6 VMEs, 5 ME, 12 mie, and 5 vmie (Table 3). Acquired resistances for
153 fluconazole were significantly more often detected by MCT than by routine AFST (34/405, 8.4%
154 versus 25/405, 6.2%, p = 0.012), while for anidulafungin, this was not statistically significant
155 (12/405, 3.0% versus 9/405, 2.2%, MCT vs. routine AFST, respectively; p = 0.453).

156 Four samples with strong PR were selected for genotyping of the five separate isolates,
157 MICs with replicates are displayed in Table 4. MLST was performed for samples I 38 (*C. albicans)*,
158 I 15 *(C. glabrata)*, and E 23 (*C. tropicalis).* In the absence of an MLST scheme for *C. lusitaniae,*
159 RAPD was performed for sample K 58.

160 In sample I 38 (*C. albicans*), isolate E displayed an elevated MIC for fluconazole (4 µg/ml)
161 compared to the other isolates (0.25-0.5 µg/ml). The obtained MLST sequences allowed
162 unambiguous determination of the sequence types (ST 1110), which was identical between the
163 isolates A-D (Table 4). For isolate E however, no clean sequence could be obtained for the locus

SYA1. All other loci were identical with the other isolates. In sample I 15 (*C. glabrata*), isolates B and D displayed an I MIC for fluconazole of 4 and 8 µg/ml, the same as obtained in routine AFST. In contrast, isolates A, C and E displayed MICs >256 in Etest©. Upon repetition by Etest© and EUCAST microdilution, isolate D showed insufficient growth for microdilution, while the remaining tests showed partly fluctuating MICs. MLST schemes for all isolates were identical (ST 3), except for B, for which no clean sequence could be obtained for the locus TRP1 (Table 4). Sample E 23 (*C. tropicalis*) showed MICs for fluconazole in the S, I and R category. However, upon repetition with Etest© and EUCAST microdilution, all MICs were in the S category (0.125-0.25 µg/ml). All isolates showed identical MLST profiles, however without an existing match in the database (Table 4). In sample K 58, isolate A had shown macrocolonies for fluconazole Etest©, which upon repetition showed a MIC of 4 µg/ml in Etest© and 8 µg/ml in EUCAST microdilution. With primers OPA-1 and OPA-8, RAPD bands differed between isolate A and B-D (Figure 1).

Altogether, PR occurred in up to 8.1% (33/405) of samples replicated by MCT, with higher rates for non-*albicans* species than for *C. albicans*. Strong PR was observed in 2% (8/405) of samples, again with higher rates in non-*albicans* species. Genotyping of selected isolates showed that the reasons for PR were manifold.

Discussion

PR, here defined as heterogeneous results in AFST of five different colonies from a same-species *Candida* sample, was observed in 33 of 405 samples (8.1%). Under ideal conditions to reduce biological and methodological variation, repeated testing of the same *Candida* WT isolate produced MIC values within a three log2 dilution range [24]. Hence, we defined a three log2

dilution range within the same EUCAST category as an accepted technical variance. MICs exceeding this range or differences in categories (EUCAST S, I, R or WT, non-WT) were defined as PR. Strong PR with categorically (EUCAST S, I, R) different MICs above three log2 dilutions is clearly the most pronounced form of PR and occurred with a rate of 2% across all species. In clinical practice, moderate PR may have the same implications for patient management as strong PR, despite smaller differences in MICs. Contrarily, weak PR may not influence patient management, but nevertheless questions the reliability of AFST. Moderate PR with borderline MICs in different EUCAST categories less than three log2 dilutions apart, was more common for fluconazole than for anidulafungin, especially with *C. glabrata* varying between I and R. Generally, the good agreement between the two methods allows translation of Etest© MICs to EUCAST breakpoints [25,26]. However, due to some methodological variance, ECVs specifically evaluated for Etest© may more sensitively identify resistances than reference BPs evaluated for microdilution [27,28]. For both fluconazole and anidulafungin, ECVs for Etest© are usually lower than EUCAST clinical breakpoints [28,29]. Especially for low-level resistances, MCT may aid disclosing otherwise undetected resistances, as some MICs will be read above the breakpoint concentration. Altogether, colonies with a categorically (EUCAST S, I, R) higher MIC than in routine AFST were found in 4.4%. Classification of MICs close to the breakpoint concentration was less consistent upon re-testing, meaning that some colonies showed MICs above and some below the breakpoint, often resulting in moderate PR. However, for samples with single deviant colonies, the MIC of the routine AFST result was not indicative of PR. Routine AFST builds the basis for decisions regarding antifungal therapy, hence fast and reliable results are necessary. Our study

206 shows that in PR, AFST results are partly incidental, potentially leading to suboptimal selection of
207 antifungal therapy or breakthrough infections.

208 Generally, non-*albicans* species show higher resistance rates than *C. albicans,* and
209 acquired resistance occurs more frequently towards azoles than towards echinocandins [2].
210 Accordingly, PR occurred in our study with higher rates in non-*albicans* species than in *C. albicans,*
211 and more frequently for fluconazole than for anidulafungin. These differences must likely be
212 considered as a product of overall resistance rates, which determine the probability of
213 encountering resistant subpopulations. These insights may aid in the consideration of ambiguous
214 AFST results in non-*albicans* species and treatment with fluconazole.

215 The presence of multiple *Candida* strains was more commonly found in feces, urine, and
216 vaginal samples than in sterile body sites [7–9], supporting the hypothesis that only a small
217 number of cells enter a sterile site of infection [8]. However, our PR samples originated from a
218 variety of infected sterile body sites, with urine and bile samples as an exception, yet usually free
219 from a colonizing *Candida* flora. Nine (5.6%) of the 161 investigated blood cultures were PR,
220 demonstrating that PR as a manifestation of intra-host diversity also occurs in sterile body
221 regions. Several lineages may co-exist or rapidly adapt under stress [9], resulting in PR upon
222 separate testing of single colonies. Accordingly, cell-to-cell variations in drug response
223 (heteroresistance) have been described for fungi, with high rates of heteroresistance for *C.*
224 *glabrata* and fluconazole, however not for echinocandins [30]. Population analysis profiling (PAP)
225 [30] or the molecular analysis of resistance mechanisms enable an elaborate characterization of
226 the underlying reasons for some types of PR, however, these methods are not suitable for routine

application. The term PR here was defined as heterogeneous MICs from a same-species *Candida* culture, to help describe heterogeneous AFST results irrespective of the underlying mechanism.

In our study, we performed genotyping to assess the clonality of four strains with the most pronounced strong PR to address the possibility of multiple infections of the same species but differently susceptible strains. In MLST, two combinations of sample and locus repeatedly failed to give clear sequencing results for unknown reasons. Other studies demonstrated the co-existence of multiple related but divergent genotypes differing in several thousand Single nucleotide polymorphisms (SNPs) and harboring different fluconazole MICs [9]. Recent data indicate that MLST only rarely reveals this diversity, namely when an SNP affects by chance one of the MLST loci [7]. A similar background might be possible for our *C. albicans, C. glabrata* and *C. tropicalis* samples with largely identical MLST loci. Underlying might be an unknown number of SNPs becoming apparent as PR but not discovered by MLST.

The selected PR *C. lusitaniae* sample showed a reproducible MIC of 4 µg/ml for isolate A as opposed to 0.064 µg/ml for the remaining four isolates and the routine result. RAPD band patterns for isolate A differed from isolates B-E, suggesting the presence of a second *C. lusitaniae* clone in the sample. Altogether, the results of genotyping PR isolates indicate that the reasons for PR can be manifold, with no single underlying mechanism.

There are some limitations to our study. Some of the R MCT results were not confirmed by a second method. Especially for borderline R results, the technical variance between Etest© and microdilution can produce different results with another method. However, the aim of the study was to evaluate the level of variance obtained by multiple testing in a real-life setting. Furthermore, we did not investigate the underlying molecular resistance mechanisms of the PR

strains, as this would have exceeded the objective of the study. This should be elucidated in a subsequent study. Moreover, MLST as used in our study has lower discriminatory power in genotyping *Candida*, than for example whole-genome sequencing, which would detect all SNPs of diversifying lineages [7]. Differences between diverging progeny of the same strain may therefore stayed undiscovered.

In conclusion, our results demonstrate that PR is a real-life phenomenon and that one single MIC-value obtained by standard AFST may not be as reliable as expected. As a result for clinical practice, more than one colony should be included in AFST. Further research is needed about the clinical implications of this phenomenon. However, we must ultimately assume that resistances may go undetected by standard AFST, and consider applying adaptions such as MCT or modified inocula to the repertoire of AFST.

Transparency declaration

The authors received no financial support for this work and have declared no conflicts of interest exists.

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Author contributions

Conceptualization and methodology: C.L.-F. and M.A.K. Acquisition, analysis and interpretation of data: E.S., N.L, M.A.K., H.L.V., P.-M.R., J.S., F.F., P.K., A.A., and R.K. Statistical analysis: H.U. and M.A.K. Supervision: C.L.-F. Writing – original draft preparation: M.A.K., C.L.-F. Writing – review and editing: All authors.

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Figures and Tables

Fig. 1.

Band patterns obtained with primers OPA-1 and OPA-8 for *C. lusitaniae*. Bands differed between isolate A (bands 1 and 6) and isolates B-D (bands 2-5 and 7-10) for both primers. M: 1 kb marker.

Table 1

Sample origins and number of samples tested with multiple colony testing (MCT) per center. Other types of samples included puncture and drainage fluids from various sterile regions, swabs from sterile regions, gastric liquid, and wound secretions. Polyresistant samples from on site centers were sent to the central lab, where genotyping was performed for selected samples.

Table 2

Number of polyresistant samples as obtained by multiple colony testing (MCT) and PR samples by species in percent. Polyresistance (PR) was defined as heterogeneous AFST results from a same-species *Candida* sample upon re-testing of colonies. PR was divided in categorically (EUCAST S, I, R) polyresistant MICs above 3 log2 dilutions (strong PR), categorically (EUCAST S, I, R) polyresistant MICs up to 3 log2 dilutions (moderate PR) and polyresistant MICs above 3 log2 dilutions without categorical (EUCAST S, I, R) difference (weak PR). Species with only homogeneous results by MCT were summarized as other species. Other species included *C. dubliniensis* (n=16), *C. kefyr* (n=7), *C. fabianii* (n=2), *C. inconspicua* (n=2), *S. cerevisiae* (n=2), *C. metapsilosis* (n=1), *C. auris* (n=1), and *C. guilliermondii* (n=1).

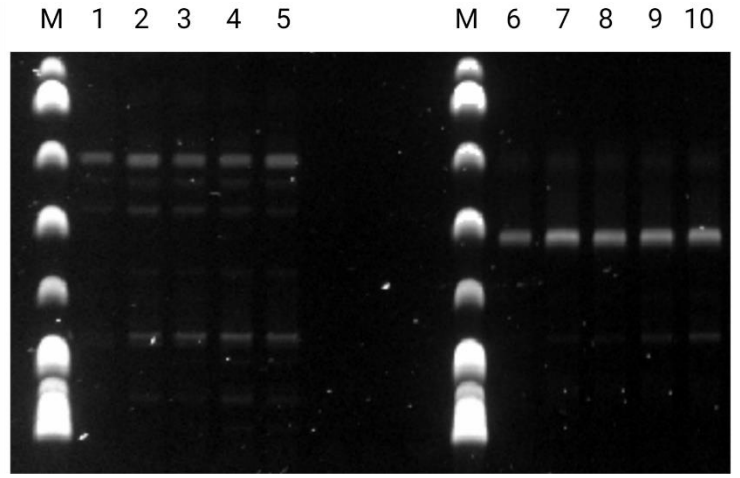
Table 3

Polyresistant isolates obtained by multiple colony testing (MCT) listed by the origin of the samples. Polyresistance (PR) was marked in categorically (EUCAST S, I, R) polyresistant MICs above three log2 dilutions (strong PR), polyresistant MICs up to three log2 dilutions (moderate PR) and polyresistant MICs above three log2 dilutions without categorical (EUCAST S, I, R) difference (weak PR). Others included sterile puncture fluids, bile, tissues, gastric liquids, wound secretions, and drainage fluids from various sterile regions.

Table 4

Multilocus sequence typing (MLST) allele types and sequence types for individual isolates from samples with strong polyresistance (PR). MICs in µg/ml obtained by MCT with Etest© are displayed on the right and marked as reproducible within ±2 log2 dilutions by Etest© and EUCAST microdilution or as not reproducible.

441 Figure 1



442

443 Table 1

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	On site centers					Central lab	
Sample type	Essen	Graz	Cologne	Nürnberg	Paris	Innsbruck	Total
Blood cultures	33	8	66	30	10	14	161
Urine	34					44	78
Intraabdominal	9	4		31		33	77
Bile		4		4		18	26
Pleural / lung				6		1	7
Catheter tip		2		1		8	11
Tissue	9			7		3	19
Others	15	2		3		6	26
Total	100	20	66	82	10	127	405
PR samples	15	1	4	5	0	8	33
Samples selected for genotyping	1	0	1	0	0	2	4

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446 Table 2

Species	Polyresistant results										Homo-genous results
	Strong PR			Moderate PR			Weak PR				
	FLU	ANI	Total	FLU	ANI	Total	FLU	ANI	Total	Total PR	
C. albicans (n=219)	2[a]		2	3	3[a]	6				7[a]	212
C. glabrata (n=91)	1		1	9	1	10	1	2	3	14	77
C. parapsilosis (n=25)		1	1	2	2	4				5	20
C. tropicalis (n=23)	2	1	3	1[a]	1[a]	1[a]				4[a]	19
C. krusei (n=13)								2	2	2	11
C. lusitaniae (n=2)	1		1							1	1
Other species										0	32
Total	6	2	8	15	7	21[a]	1	4	5	33[a]	372
C. albicans (n=219) %	0.9%	0.0%	0.9%	1.4%	1.4%	2.7%	0.0%	0.0%	0.0%	3.2%	96.8%
non-*albicans* (n=186) %	2.2%	1.1%	3.2%	6.5%	2.2%	8.6%	0.5%	2.2%	2.7%	14.0%	86.0%
Total %	1.5%	0.5%	2.0%	3.5%	1.7%	5.2%	0.2%	1.0%	1.2%	8.1%	91.9%

[a] same isolate exhibiting polyresistant results for FLU and ANI

FLU, fluconazole; ANI, anidulafungin

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Table 3

Specimen type	Polyresistance	Number of polyresistant isolates			
		C. albicans		non-*albicans*	
		FLU	ANI	FLU	ANI
Blood cultures (n=161)	Strong			1	1
	Moderate			5	2
	Weak				
Intraabdominal (n=77)	Strong			1	
	Moderate		1	2	1
	Weak				3
Urine (n=78)	Strong	1 [a]		1	1
	Moderate	2	1 [a]	5 [a]	1 [a]
	Weak				1
Others (n=89)	Strong	1		1	
	Moderate	1	1		
	Weak			1	

[a] same isolate exhibiting polyresistant results for FLU and ANI

FLU, fluconazole; ANI, anidulafungin

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451 Table 4
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C. albicans	Alleles							Sequencing type	MIC FLU	MIC Repetition Etest	MIC EUCAST microdilution	Reproducible
Isolate ID	AAT1a	ACC1	ADP1	MPIb	SYA1	VPS13	ZWF1b					
ATCC 90028	5	3	5	9	2	25	12	/	n.a.	n.a.	n.a.	n.a.
I 38 A	13	10	15	6	7	32	15	1110	0.25	0.25	0.125	yes
I 38 B	13	10	15	6	7	32	15	1110	0.5	0.125	0.125	yes
I 38 C	13	10	15	6	7	32	15	1110	0.5	0.25	0.125	yes
I 38 D	13	10	15	6	7	32	15	1110	0.5	0.125	0.125	yes
I 38 E	13	10	15	6	/	32	15	/	4	4	2	yes

C. glabrata	Alleles						Sequencing type	MIC FLU	MIC Repetition Etest	MIC EUCAST microdilution	Reproducible
Isolate ID	FKS	LEU2	NMT1	TRP1	UGP1	URA3					
ATCC 90030	8	4	3	5	1	2	10	n.a.	n.a.	n.a.	n.a.
I 15 A	5	7	8	7	3	6	3	>256	64	32	no
I 15 B	5	7	8	/	3	6	/	4	8	8	yes
I 15 C	5	7	8	7	3	6	3	>256	128	32	no
I 15 D	5	7	8	7	3	6	3	8	8	x	no
I 15 E	5	7	8	7	3	6	3	>256	128	32	no

C. tropicalis	Alleles						Sequencing type	MIC FLU	MIC Repetition Etest	MIC EUCAST microdilution	Reproducible
Isolate ID	ICL1	MDR1	SAPT2	SAPT4	XYR1	ZWF1a					
ATCC 90874	1	1	11	9	77	1	/	n.a.	n.a.	n.a.	n.a.
E 23 A	1	1	10	1	73	1	/	4	0.125	0.125	no
E 23 B	1	1	10	1	73	1	/	8	0.25	0.125	no
E 23 C	1	1	10	1	73	1	/	0.5	0.25	0.125	yes
E 23 D	1	1	10	1	73	1	/	4	0.125	0.125	no
E 23 E	1	1	10	1	73	1	/	8	0.125	0.25	no

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454 FLU, fluconazole