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Identification and antifungal susceptibility patterns of reference yeast strains to novel and conventional agents: a comparative study using CLSI, EUCAST and Sensititre YeastOne methods

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Objectives: The aim of this study was to identify and determine the MICs of 13 antifungal drugs, including the novel agents ibrexafungerp, manogepix and rezafungin, against 22 laboratory reference strains from 14 different *Candida* spp. and allied yeast genera using the EUCAST, CLSI and Sensititre™ YeastOne™ (SYO) methods.

Results: Complete agreement between molecular and proteomics methods was observed for identification. The compounds with the greatest *in vitro* activity, as indicated by the lowest geometric mean MIC (GM), were manogepix (GM: 0.01), isavuconazole (GM: 0.05) and rezafungin (GM: 0.03–0.07). The overall essential agreement (EA) (within ±0 to ±2 2-fold dilutions) between the reference methods, EUCAST and CLSI, was 95%, with results ranging from 82% (ibrexafungerp) to 100% (amphotericin B, anidulafungin, fluconazole, 5-flucytosine and micafungin). Regarding EA for EUCAST and CLSI compared with SYO, values were 91% and 89%, respectively. Nevertheless, when the MIC values were transformed into log2, significant differences were observed (e.g. fluconazole, ibrexafungerp and 5-flucytosine). At the species level, *Candidozyma auris* and *Candida duobushaemulonii* exhibited the highest number of cases with significant differences when comparing the three techniques for each antifungal.

Conclusions: The high EA observed reinforces the reliability of EUCAST, CLSI and SYO in guiding antifungal therapy. However, the differences in EA, particularly for ibrexafungerp and 5-flucytosine, highlight the importance of continued evaluation of these methodologies to ensure consistency. Given that antifungal susceptibility testing plays a critical role in treatment decisions, understanding these variations is essential to prevent potential misclassification of susceptibility profiles, which could impact clinical outcomes.

Introduction

Antifungal susceptibility testing (AFST) is a key tool in the management of fungal infections, guiding the selection of appropriate antifungal therapy. Additionally, AFST provides important information for ecological and epidemiological issues.[1,2] A crucial component of AFST is the use of reference yeast strains, which serve as globally recognized benchmarks in laboratories for both clinical and research purposes.[3,4] These strains provide a consistent standard against which the efficacy of antifungal agents can be measured, making the understanding of their susceptibility profiles crucial. Such profiles serve as reference points for interpreting results and ensuring accuracy and reproducibility across different laboratories and studies.[5]

Discrepancies between MIC values and clinical outcomes can arise due to variations in testing methods. The MIC values can differ depending on the AST method used.[6] In addition to the broth microdilution (BMD) reference method recommended by the CLSI and EUCAST, there are several other methods for determining MICs, for instance, colorimetric-based assays [e.g. Sensititre YeastOne™ (SYO) and MICRONAUT-AM], agar-based methods employing concentration gradients of antifungals such as epsilometer test (Etest) and automated systems (e.g. Vitek™ 2 system), each of these methods offers distinct

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advantages and limitations, influencing their adoption in clinical laboratories.[7]

In addition to CLSI and EUCAST, the SYO method was included in this study due to its widespread use in clinical laboratories as a standardized, commercially available AFST system. Its advantages, such as ease of implementation, colorimetric endpoint determination and compatibility with routine workflows, make it a valuable alternative for AFST.[8] Evaluating its concordance with reference methods is crucial for assessing its reliability in clinical settings.

To date, few studies have compared the MICs of reference yeast strains by different AFST methods.[9–11] In addition, comparison of the activity of novel antifungal agents, such as ibrexafungerp, manogepix and rezafungin, by reference methods has not been performed on those strains. This study addresses these gaps by comparing AFST methods with the inclusion of new molecules against globally recognized reference strains of the order Saccharomycetales, including those previously classified in the genus *Candida* (such as *Candida albicans*) and those reclassified into new genera such as *Nakaseomyces glabratus* (formerly *Candida glabrata*), *Clavispora lusitaniae* (formerly *Candida lusitaniae*), *Meyerozyma guilliermondii* (formerly *Candida guilliermondii*) and *Pichia kudriavzevii* (formerly *Candida krusei*).[12]

The increasing incidence of antifungal resistance among clinically relevant yeasts, particularly in species such as *Candidozyma auris* (*Ca. auris*) and *C. glabrata*, has become a major concern in global healthcare.[13,14] The emergence of resistance to multiple drug classes, including azoles and echinocandins, highlights the need for accurate and standardized susceptibility testing methods. This study addresses this urgent issue by evaluating both conventional and novel antifungal agents using different AFST methodologies to ensure consistency in MIC determination and improve therapeutic decision-making.

Materials and methods

Isolates and identification

A total of 22 laboratory reference strains obtained from the ATCC, the Deutsche Sammlung von Mikroorganismen (DSMZ; Braunschweig, Germany), UKHSA's National Collection of Pathogenic Fungi (NCPF, UK) or the Centraalbureau voor Schimmelcultures (CBS) culture collection hosted at the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) were used: *C. albicans* ATCC 10231, *C. albicans* ATCC 90028, *C. albicans* SC5314/MYA-2876, *Ca. auris* CBS 16214, *Ca. auris* DSM 105988 (South Africa-Clade III)/CDC AR-Bank 0383, *Ca. auris* DSM 105990 (South America-Clade IV)/CDC AR-Bank 0385, *Ca. auris* DSM 105992 (South Asia-Clade I)/CDC AR-Bank 0387, *Ca. auris* DSM 21092 (East Asia-Clade II)/CBS 10913, *Candida blankii* NCPF 8710, *Candida dubliniensis* DSM 28723, *Candida duobushaemulonii* DSM 105996, *C. glabrata* (*N. glabratus*) ATCC 2001, *C. glabrata* (*N. glabratus*) CBS 15716, *C. guilliermondii* (*M. guilliermondii*) ATCC 7350/DSM6260, *Candida haemulonii* DSM 105998/CDC AR-Bank 0393, *C. krusei* (*P. kudriavzevii*) ATCC 6258, *Cl. lusitaniae* (*C. lusitaniae*) DSM 70102/CBS 5094, *Candida metapsilosis* NCPF 8790, *Candida orthopsilosis* DSM 24508, *Candida parapsilosis* ATCC 22019/CBS 604, *C. parapsilosis* CBS1954 and *C. tropicalis* ATCC 750. The 22 yeast strains included in this study were selected based on the available resources allocated for the establishment of the new mycology laboratory. Given these constraints, we prioritized species that are most studied in clinical mycology and those classified as emerging yeasts with potential clinical significance. The strain collection was preserved at −80°C (glycerol stocks). In the week of the experiments, the isolates were inoculated onto Sabouraud Dextrose Agar plates and chromogenic media, i.e. CHROMagar™ Candida Plus® (CCP) (CHROMagar, Paris, France) and chromID™ Candida (CID) (BioMérieux, Marcy-l'Étoile, France) followed by incubation at 35°C for 24–36 h. The identity of all strains was confirmed by molecular and proteomic-based methods. For proteomic identification, the Vitek MS analysis was performed according to the manufacturer's instructions. Briefly, a small portion of a single colony was directly spotted onto a target plate and covered with 0.5 μL formic acid (bioMérieux) and allowed to dry prior to being loaded into the Vitek MS system (bioMérieux, Inc.). A strain was considered correctly identified with an acceptable confidence value of ≥99%. For molecular means, DNA sequencing of the fungal internal transcribed spacer (ITS) region obtained from direct colony PCR was carried out, a single colony of approximately 1 mm in diameter was suspended in 50 μL of sterile distilled water and heated at 95°C for 10 min, then 5 μL were used for PCR.[15] Universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′).[16] were used to amplify the ITS region under the following conditions: denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 60 s and elongation at 72°C for 60 s, with a final extension step of 10 min at 72°C. Nucleotide sequences were compared with reference data available in GenBank, using the BLAST algorithm or with the MycoBank database. A similarity ≥99% between the unknown sequence and the closest matching sequence from the reference database was used as the criterion to identify an isolate to the species level.[17]

Antifungal susceptibility testing

Reference AFST was carried out following the CLSI-M27 4th ed[18] and the EUCAST E.Def 7.4[19] guidelines. Antifungal drugs included amphotericin B, anidulafungin, caspofungin, fluconazole, flucytosine, ibrexafungerp, isavuconazole, itraconazole, manogepix, micafungin, posaconazole, rezafungin (0.002% Tween 20-supplemented) and voriconazole.

The antifungal drugs were purchased from Sigma-Aldrich (France) or obtained from their manufacturers as standard powders. Drug-free and yeast-free controls were included, and microtiter plates [Thermo Scientific™ Nunc™ 96-Well, Nunclon Delta-Treated, Flat-Bottom REF 10567131 and Round Bottom (U) REF 10344311 microplates] were incubated at 35°C and read visually for the CLSI method and spectrophotometrically (OD at 530 nm) for the EUCAST method after 24 h.

The MIC endpoints for azoles, echinocandins and manogepix were defined as the lowest drug concentration that caused a prominent decrease in visual growth (CLSI) or a reduction to ≤50% of growth (EUCAST) in comparison with the control-well containing media only. For amphotericin B, the MIC was defined as the lowest concentration at which there was full inhibition of visual growth (CLSI) or a reduction to ≤10% of growth (EUCAST) relative to the drug-free control wells. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 strains were used as quality control strains.[20,21] For ibrexafungerp assays, the strain *C. albicans* ATCC 90028 was used as quality control.[20]

AFST was also performed by Sensititre YeastOne AST YO10 plate (TREK Diagnostic Systems, Cleveland, OH, USA), following manufacturers' instructions. Briefly, the dried SYO panels were rehydrated with the yeast suspension using an appropriate multichannel pipetting device by dispensing 100 μL into each well. Panels were covered with adhesive seals and incubated at 35°C for 24 h. MICs endpoints were read visually under normal laboratory lighting after 24 h of incubation. Evident yeast growth was observed as the colour changed from blue (negative, indicating no growth) to pink (positive, indicating growth). Susceptibility to amphotericin B, anidulafungin, caspofungin, fluconazole, itraconazole, micafungin, posaconazole and voriconazole was evaluated by using the colorimetric microdilution panels. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 strains were used as quality control strains. All experiments were conducted in triplicate; however, in this study, only one value is presented

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for each case, either the value that appeared most frequently among the three replicates or the one closest to the mean. This approach was adopted to align with the measurement criteria recommended by CLSI or EUCAST for susceptibility studies.

Data analysis

MIC ranges and geometric mean MIC (GM) values were calculated for each drug and method. High off-scale MIC results were converted to the next highest concentration, and low off-scale MIC results were left unchanged. Essential agreement (EA) was considered as discrepancies in MIC results of no more than ±2 serial 2-fold dilutions.[22]

The MIC values obtained for the 22 strains evaluated using the CLSI, EUCAST and SYO methods, were statistically analysed to identify significant differences across techniques using GraphPad Prism v10 (GraphPad Software, San Diego, CA, USA). To address data skewness and stabilize variances, MIC values were log2-transformed. Normality was assessed using the Shapiro–Wilk test, with a P value ≥ 0.05 indicating normality. When log2-transformed data met normality assumptions, comparisons between two techniques were conducted using a paired *t*-test, while comparisons among three techniques were performed using a two-way repeated-measures ANOVA, considering the MIC determination method as the repeated-measures factor and fungal species as an independent factor. When significant differences were detected, *post hoc* pairwise comparisons were performed using Tukey's test. If normality was not met, non-parametric tests were applied: the Wilcoxon signed-rank test for two techniques and the Friedman test for three techniques, followed by Wilcoxon *post hoc* comparisons with Bonferroni correction. Potential outliers were identified using Grubbs' test and were carefully reviewed to determine whether they represented experimental errors or valid biological variability. Data visualization included dot plots to compare MIC distributions across methods and Q–Q plots (Figure S1, available as Supplementary data at *JAC-AMR* Online) to evaluate normality.

Results

Yeast identification

In the process of establishing a new medical mycology laboratory, molecular and proteomic methodologies were developed for yeast identification. These methods were subsequently used to confirm the identification of the strains. All 22 yeast strains were accurately identified using both molecular biology (PCR and sequencing of ITS region) and Vitek MS techniques, achieving a 100% concordance rate between the two methods. Furthermore, all reference strains showed growth in the two chromogenic media used (intended solely to confirm strain purity), with visible colonies observed after 24–36 h of incubation. Figure 1 shows the variety in morphology and pigmentation of colonies after 36 h incubation. All strains, except for one (*C. parapsilosis* CBS2195, which was excluded from this study), were pure, with no contamination or mixed phenotypes observed. The phenotypes matched those described for each species. CCP showed the best capacity to discriminate among *Candida* species, with *Ca. auris*, *C. orthopsilosis* and *C. tropicalis* displaying unique morphological features. Additionally, *C. albicans* exhibited a distinct pigmentation compared with most other *Candida* species, except for the closely related *C. dubliniensis*, which showed a similar pigmentation pattern. Other species than those mentioned above were not reliably distinguishable using CCP.

Antifungal susceptibility testing

The MICs determined using the reference CLSI, EUCAST and commercial SYO methods for azoles and amphotericin B antifungal compounds against all reference yeast strains are displayed in Table 1, Figure 2. Results for echinocandins and the remaining

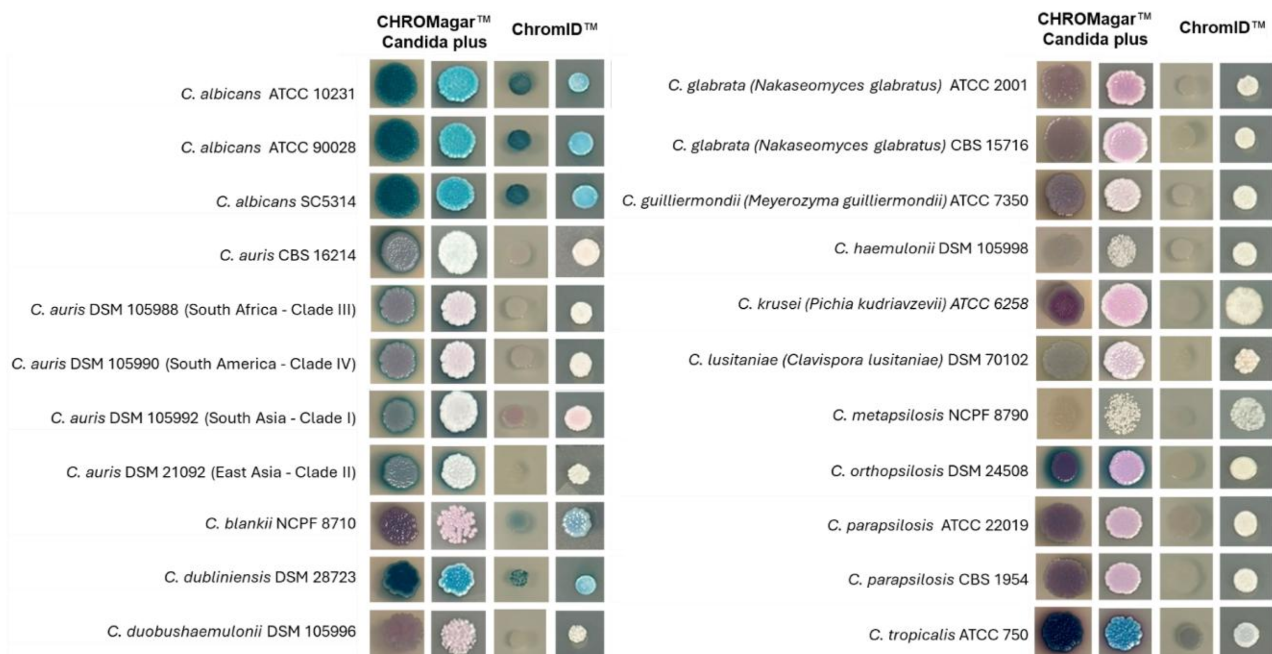

Figure 1. Comparative colonial appearances of common species of *Candida* and allied yeast genera spotted onto CHROMAgar™ Candida Plus and ChromID™. Photographs were taken on white (left) and black (left) backgrounds to improve the visualization of pigmentation.

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Table 1. In vitro susceptibilities of 22 reference yeast strains to azoles and amphotericin B agents as determined by CLSI, EUCAST and SYO

Drug	MIC (mg/L)															
	FLC			ISA	ITC			POS			VRC			AMB		
Strain/Method	EUCAST	CLSI	SYO	CLSI	EUCAST	CLSI	SYO	EUCAST	CLSI	SYO	EUCAST	CLSI	SYO	EUCAST	CLSI	SYO
C. albicans ATCC10231	0.5	0.5	1	<0.03	0.06	0.12	0.06	0.06	**0.12**	**0.12**	0.016	<0.03	0.03	0.25	0.25	0.5
C. albicans ATCC90028	0.25	0.5	0.5	<0.03	0.03	0.12	0.12	0.03	**0.12**	**0.06**	0.008	<0.03	0.016	0.25	0.5	0.5
C. albicans SC5314	2	0.5	0.5	<0.03	0.06	0.12	0.12	0.03	**0.12**	**0.06**	0.03	<0.03	0.016	0.25	0.5	0.5
Ca. auris CBS16214	**>32**	**64**	**128**	0.06	0.25	0.12	0.25	0.12	0.06	0.12	0.25	0.5	1	**2**	**2**	**4**
Ca. auris DSM105988	**>32**	**>64**	**256**	0.06	1	0.5	0.5	0.12	0.25	0.25	4	2	2	0.5	0.5	1
Ca. auris DSM105990	**>32**	**>64**	**256**	0.5	0.5	0.25	0.5	0.5	0.25	0.25	4	8	8	0.5	1	**2**
Ca. auris DSM105992	4	2	2	<0.03	2	0.12	0.12	0.12	0.06	0.06	0.25	0.03	1	0.5	1	1
Ca. auris DSM21092	8	4	2	0.03	0.12	0.06	0.12	0.06	0.03	0.06	0.06	<0.03	0.03	0.5	1	0.5
C. blankii NCPF8710	16	4	4	0.06	0.5	0.25	0.25	1	0.25	0.5	0.25	0.25	0.25	0.25	1	0.5
C. dubliniensis DSM28723	0.25	0.12	0.25	<0.03	0.25	0.12	0.06	1	0.06	0.06	0.06	<0.03	0.008	0.12	0.25	0.12
C. duobushaemulonii DSM105996	16	4	8	0.03	0.5	0.25	0.25	4	0.12	0.12	4	0.12	0.06	2	2	0.5
C. glabrata ATCC2001	4	4	16	0.06	0.25	0.5	1	0.5	0.25	**2**	0.12	0.12	**0.25**	0.5	0.5	0.5
C. glabrata CBS15716	4	2	32	0.03	0.5	0.25	1	0.5	0.25	**2**	0.12	0.06	**0.25**	1	1	1
C. guilliermondii ATCC7350	1	1	2	<0.03	0.12	0.12	0.25	0.12	0.06	0.25	0.03	<0.03	0.03	0.25	0.25	<0.12
C. haemulonii DSM105998	4	2	16	0.12	0.5	0.25	0.5	0.25	0.12	0.25	0.12	0.06	0.25	2	2	2
C. krusei ATCC6258	16	16	32	0.06	0.12	0.12	0.25	0.12[a]	0.12	0.25	0.12	0.12	0.12	0.5	1	0.5
Cl. lusitaniae DSM70102	0.5	0.5	**1**	0.03	0.06	0.12	0.12	0.06	**0.06**	0.03	0.016	0.03	0.016	0.25	0.5	0.25
C. metapsilosis NCPF8790	1	1	2	<0.03	0.12	0.12	0.06	0.06	0.06	0.03	0.03	<0.03	0.03	0.12	0.5	0.25
C. orthopsilosis DSM24508	0.25	0.25	1	0.03	0.25	0.12	0.12	0.12	0.06	0.12	0.03	<0.03	0.03	0.25	0.5	0.25
C. parapsilosis ATCC22019	1	1	2	0.06	0.12	0.25	0.25	0.03	0.12	0.25	0.03	0.03	0.03	0.5	1	0.25
C. parapsilosis CBS1954	1	0.5	2	0.03	0.06	0.25	0.03	0.06	0.06	0.06	0.03	<0.03	0.016	0.25	0.5	0.25
C. tropicalis ATCC750	1	0.25	2	0.06	0.12	0.06	0.25	0.12	0.06	**0.12**	0.06	0.03	0.12	1	0.5	0.5
Range	0.06–32	0.125–64	0.12–256	0.03–16	0.008–4	0.03–16	0.016–16	0.008–4	0.03–16	0.008–8	0.008–4	0.03–16	0.008–8	0.008–4	0.03–16	0.12–8
Mean	12	19	35	0.07	0.3	0.2	0.3	0.4	0.12	0.3	0.6	0.4	0.6	0.6	0.8	0.8
GM	2.92	2.49	4.68	0.05	0.20	0.16	0.19	0.16	0.10	0.15	0.10	0.08	0.09	0.43	0.69	0.51

Strains considered resistant according to CLSI M27M44S-Ed3, M57S-Ed4, tentative CDC breakpoints (*Ca. auris*) or EUCAST E.Def 7.4-V4.0 are shown in bold.
ISA, isavuconazole; GM, geometric mean MIC.
[a]Value out of range.

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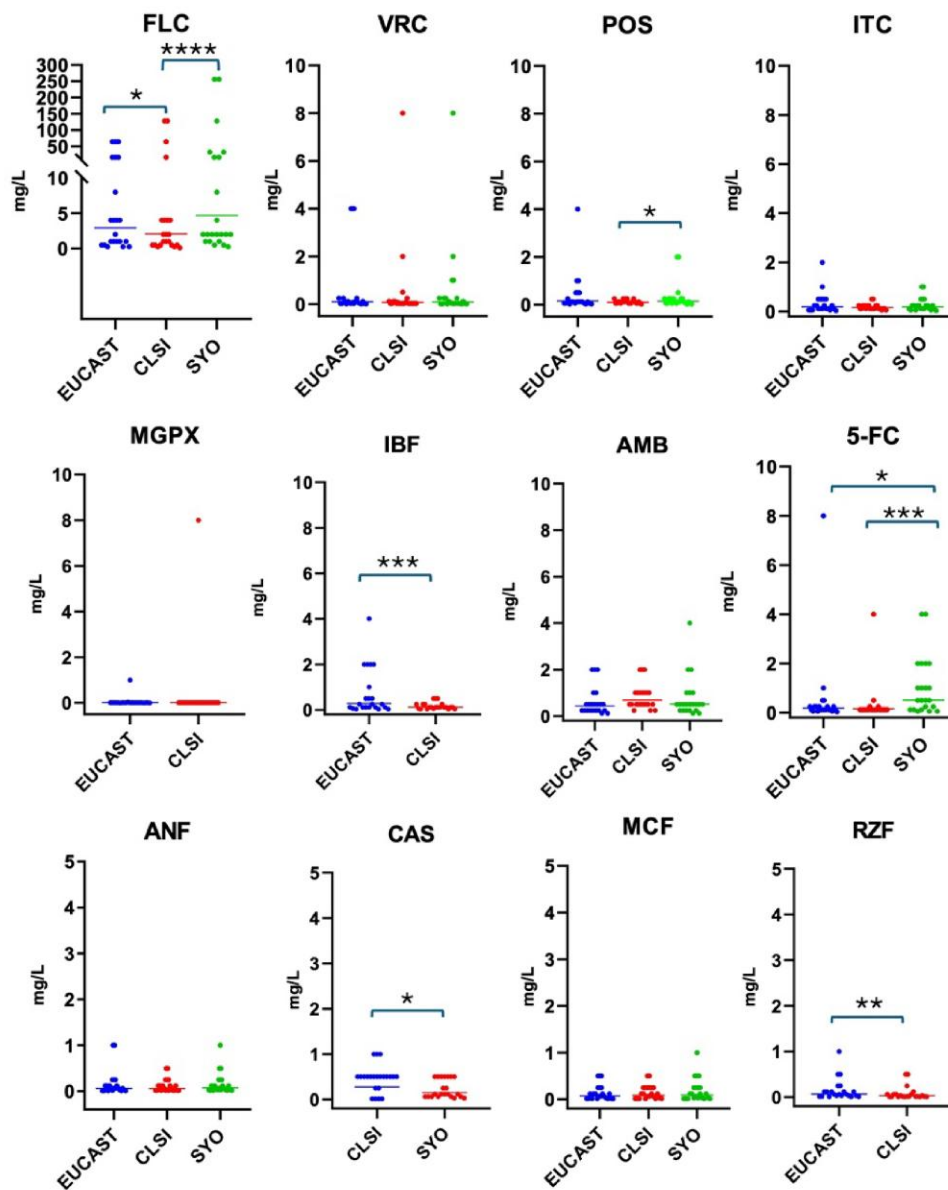

Figure 2. Distribution of MIC values for 22 strains evaluated by CLSI, EUCAST SYO methods for each antifungal agent. Each dot represents the consensus MIC value (measured in triplicate) for a single strain. The horizontal line within each method's distribution corresponds to the GM MIC. FLC, fluconazole; VRC, voriconazole; POS, posaconazole; ITC, itraconazole; MGPX, manogepix; IBF, ibrexafungerp; AMB, amphotericin B; 5FC, flucytosine; AFG, anidulafungin; CAS, caspofungin; MFG, micafungin; and RZF, rezafungin. * ≤ 0.0332, ** ≤ 0.0021, *** ≤ 0.0002, **** ≤ 0.0001.

antifungal agents are presented in Table 2, Figure 2. All MIC values for the control strains, except for two cases (*C. krusei* with posaconazole and 5-flucytosine, EUCAST, which were one dilution apart), fall within the cut-off range established by CLSI M27M44S-Ed3 and EUCAST v 6.0 guidelines. Strains obtained from the CDC AR-Bank also fell within the accepted range (±2 serial 2-fold dilutions; https://wwwn.cdc.gov/ARIsolateBank/Panel/PanelDetail?ID=2). The antifungal agents demonstrated varying degrees of activity against the tested yeast strains. The compounds with the greatest *in vitro* activity, as indicated by the lowest GM MIC, were manogepix (GM: 0.01), isavuconazole (GM: 0.05) and rezafungin (GM: 0.03–0.07). In contrast, the compound with the least *in vitro* antifungal activity was fluconazole (GM: 2.49–4.68). The MIC values obtained using the CLSI method were generally lower than those determined by the EUCAST method for most antifungal agents. However, an exception was observed with amphotericin B, where the MIC values from the EUCAST method were typically one or two doubling dilutions lower than those from the CLSI method. A similar pattern was noted when comparing CLSI MICs with those from the SYO method; for most agents, CLSI results were lower, except in the cases of amphotericin B and caspofungin.

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Table 2. In vitro susceptibilities of 22 reference yeast strains to echinocandins, flucytosine and novel antifungal agents as determined by CLSI, EUCAST and SYO

Drug	MIC (mg/L)																
	AFG			CAS		MFG			RZF		IBF		MGPX		5-FC		
Strain/Method	EUCAST	CLSI	SYO	CLSI	SYO	EUCAST	CLSI	SYO	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI	SYO
C. albicans ATCC10231	0.008	<0.016	0.016	<0.016	0.03	0.008	0.016	0.016	0.008	<0.008	0.03	0.03	0.008	<0.008	0.12	0.12	0.12
C. albicans ATCC90028	0.008	<0.016	0.03	<0.016	0.06	0.016	0.016	0.016	0.008	<0.008	0.03	0.03	0.008	0.008	0.5	0.12	0.12
C. albicans SC5314	0.008	<0.016	0.016	0.016	0.12	0.016	0.016	0.03	0.008	<0.008	0.03	0.03	0.004	<0.008	0.12	0.12	0.06
Ca. auris CBS16214	0.06	0.06	0.25	0.25	0.5	0.12	0.25	0.25	0.03	0.06	0.5	0.25	0.008	0.008	0.12	<0.12	2
Ca. auris DSM105988	0.12	0.25	0.25	1	0.5	0.25	0.25	0.25	0.12	0.03	2	0.25	0.016	0.016	1	0.5	4
Ca. auri DSM105990	0.06	0.25	0.12	0.5	0.5	0.25	0.25	0.25	0.12	0.06	2	0.25	0.016	0.016	0.5	0.25	0.5
Ca. auris DSM105992	0.12	0.12	0.12	0.5	0.25	0.12	0.25	0.12	0.12	0.03	2	0.25	0.008	0.008	0.12	0.25	0.06
Ca. auris DSM21092	0.06	0.016	0.03	0.5	0.06	0.06	0.06	0.06	0.03	0.016	0.5	0.12	0.008	0.008	0.25	0.12	0.5
C. blankii NCPF8710	0.25	0.12	0.12	0.5	0.12	0.12	0.12	0.12	0.12	0.016	0.12	0.12	0.008	<0.008	0.12	<0.12	2
C. dubliniensis DSM28723	0.008	<0.016	0.03	0.016	0.06	0.016	0.016	0.016	0.06	0.008	0.06	0.03	0.008	<0.008	0.06	<0.12	1
C. duobushaemulonii DSM105996	0.12	0.06	0.016	**0.5**	0.03	0.12	0.12	0.03	0.12	0.12	4	0.5	0.002	<0.008	0.25	<0.12	2
C. glabrata ATCC2001	0.016	0.016	0.06	0.25	0.06	0.016	0.016	0.016	0.016	0.008	0.12	0.12	0.03	0.016	0.06	<0.12	0.25
C. glabrata CBS15716	0.03	0.016	0.03	**0.5**	0.06	0.016	0.016	0.016	0.03	0.016	0.12	0.12	0.016	0.008	0.12	0.12	0.5
C. guilliermondii ATCC7350	0.25	0.12	0.5	0.5	0.25	0.12	0.12	0.25	0.5	0.25	0.5	0.12	0.008	0.008	0.12	<0.12	0.25
C. haemulonii DSM105998	0.06	0.03	0.016	0.5	0.12	0.06	0.06	0.12	0.06	0.016	0.25	0.12	0.008	0.008	0.12	<0.12	1
C. krusei ATCC6258	0.06	0.12	0.03	0.5	0.5	0.06	0.25	0.12	0.06	0.016	1	0.5	>0.5	>4	8[a]	4	4
Cl. lusitaniae DSM70102	0.03	<0.016	0.06	0.5	0.06	0.03	0.06	0.03	0.016	0.008	2	0.5	0.016	0.016	0.03	<0.12	2
C. metapsilosis NCPF8790	0.12	0.12	0.12	**0.5**	0.12	0.25	0.12	0.5	0.25	0.06	0.12	0.06	0.004	0.008	0.25	<0.12	0.06
C. orthopsilosis DSM24508	0.25	0.5	1	**1**	0.5	0.5	0.25	0.5	0.5	0.5	0.25	0.06	0.016	0.008	0.25	0.12	1
C. parapsilosis ATCC22019	1	0.25	0.5	0.5	0.5	0.5	0.5	1	1	0.5	0.25	0.12	0.016	0.008	0.25	0.12	0.5
C. parapsilosis CBS1954	1	0.5	0.25	1	0.5	0.5	0.5	0.5	0.25	0.06	0.12	0.12	0.016	0.008	0.25	<0.12	0.12
C. tropicalis ATCC750	0.008	0.016	0.06	0.5	0.12	0.016	0.03	0.03	0.03	0.016	0.12	0.12	0.008	0.008	0.06	<0.12	1
Range	0.008–4	0.016–8	0.016–8	0.016–8	0.008–8	0.008–4	0.016–8	0.008–8	0.008–4	0.008–4	0.016–8	0.016–8	0.001–0.5	0.008–4	0.03–16	0.12–64	0.06–64
Mean	0.2	0.1	0.2	0.5	0.2	0.2	0.1	0.2	0.2	0.09	0.7	0.2	0.06	0.4	0.4	0.3	0.9
GM	0.06	0.05	0.08	0.28	0.15	0.08	0.08	0.09	0.07	0.03	0.28	0.12	0.01	0.01	0.18	0.16	0.5

Strains considered resistant according to CLSI M27M44S-Ed3, M57S-Ed4 or EUCAST E.Def 7.4-V4.0 documents are shown in bold.
[a]Value out of range.

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Table 3. Quantitative agreement among MIC results via CLSI, EUCAST and SYO methods

	EUCAST versus CLSI				EUCAST versus SYO				CLSI versus SYO			
	Agreement within 2-fold dilutions			EA %	Agreement within 2-fold dilutions			EA%	Agreement within 2-fold dilutions			EA%
Drug	0	±1	±2		0	±1	±2		0	±1	±2	
AMB	8	12	2	100	9	10	3	100	7	13	2	100
AFG	4	15	3	100	3	10	8	95	9	7	6	100
CAS	—	—	—	—	—	—	—	—	3	7	7	77
FLC	10	8	4	100	1	12	8	95	4	12	4	91
5-FC	9	11	2	100	2	6	6	64	3	6	3	55
ISA	—	—	—	—	—	—	—	—	—	—	—	—
ITC	3	16	2	95	5	13	3	95	9	10	2	95
MFG	5	15	2	100	11	10	1	100	12	6	3	95
POS	3	13	4	91	7	10	3	91	7	12	1	91
VRC	11	8	1	91	7	11	2	91	9	9	3	95
IBF	8	6	4	82	—	—	—	—	—	—	—	—
MGPX	13	7	1	95	—	—	—	—	—	—	—	—
RZF	5	9	6	91	—	—	—	—	—	—	—	—
Overall				95				91				89

EA was considered as discrepancies in MIC results of no more than ±2 serial 2-fold dilutions.
ISA, isavuconazole; —, not applicable.

When the MIC values obtained for the novel molecules rezafungin, ibrexafungerp and manogepix were analysed (average MICs for each species) against species with more than one strain, i.e. *C. albicans* ($n=3$), *Ca. auris* ($n=5$), *C. glabrata* ($n=2$) and *C. parapsilosis* ($n=2$), antifungal activity varied depending on both the species and the antifungal. *C. parapsilosis* exhibited the highest MIC values against rezafungin, *Ca. auris* against ibrexafungerp and *C. krusei* against manogepix. Conversely, *C. albicans* consistently showed the lowest MIC values for both novel and most of the conventional molecules tested. Similarly, the *C. dubliniensis* strain showed low MIC values for most of the drugs evaluated. As expected, *Ca. auris* and *C. krusei* had the highest MICs values.

Table 3 shows the EA (within ±2 dilutions) among CLSI, EUCAST and SYO for each drug tested. The EA across methods for all drugs was >90%, except between CLSI and EUCAST for ibrexafungerp (82%), EUCAST, CLSI and SYO for 5-flucytosine (64% and 55%, respectively) and CLSI and SYO for caspofungin (77%). The highest concordance was observed between the EUCAST and CLSI methods, with an agreement rate of 95%, indicating a strong consistency in their MIC determinations. Followed by EUCAST and SYO methods with a concordance of 91%, and the CLSI and SYO methods with a concordance rate at 89%, suggesting some variability in the MIC values obtained by these methods. Remarkably, a 100% agreement was observed between the EUCAST and CLSI methods for several antifungal agents, including amphotericin B, anidulafungin, fluconazole, 5-flucytosine and micafungin. Moreover, the EUCAST and SYO methods also demonstrated complete EA for amphotericin B and micafungin. Similarly, the CLSI and SYO methods exhibited 100% EA for amphotericin B and anidulafungin. Among the tested drugs, amphotericin B was the only one showing consistent 100% EA across all methods. Additionally, manogepix was the molecule for which the highest number of strains (13 out of 22) presented the same MIC value (CLSI versus EUCAST). When the MIC values were transformed into log2, significant differences were observed for fluconazole, ibrexafungerp, 5-flucytosine, caspofungin and rezafungin (Table 4). Although good EA was observed for certain antifungals, such as fluconazole, significant differences emerged during statistical analysis. This is not unexpected, as EA considers a range of ±2 dilutions, whereas statistical analyses account for these differences more precisely. Fluconazole and 5-fluorocytosine were the antifungals with the highest variability between techniques. At the species level, *Ca. auris* and *C. duobushaemulonii* exhibited the highest number of cases with significant differences when comparing the three techniques (CLSI, EUCAST and SYO) for each antifungal. In contrast, *C. albicans* showed the fewest cases of significant differences between methods for the antifungals that displayed overall variability.

Discussion

The ability to accurately assess AFST of *Candida* species and other yeasts is crucial in both clinical and research laboratory settings. Evaluating their reliability facilitates the identification of both consistencies and discrepancies in antifungal activity across different methods, which is particularly important when AFST results are used to guide therapeutic decisions. This capability is increasingly vital given the rising incidence of fungal infections, the expanding armamentarium of antifungal agents, and the emergence of antifungal resistance as significant clinical and environmental challenges.[14,23–25] To the best of our knowledge, this study is the first to evaluate both conventional and novel antifungal agents against a set of laboratory reference strains, offering

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Table 4. Significant differences in MIC values among antifungal agents for the 22 strains evaluated in triplicate

		FLC		IBF		5-FC		CAS		RZF	
Strains	Method/drug	Adjusted *P* value		Adjusted *P* value		Adjusted *P* value		Adjusted *P* value		Adjusted *P* value	
C. albicans	EUCAST versus SYO	****	<0.0001	—	—	ns	>0.9999	—	—	—	—
ATCC10231	CLSI versus SYO	****	<0.0001	—	—	ns	>0.9999	ns	0.9684	—	—
C. albicans	EUCAST versus CLSI	****	<0.0001	ns	>0.9999	****	<0.0001	—	—	ns	>0.9999
ATCC90028	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
C. albicans	EUCAST versus CLSI	****	<0.0001	ns	>0.9999	ns	>0.9999	—	—	ns	>0.9999
SC5314	EUCAST versus SYO	****	<0.0001	—	—	ns	0.1005	—	—	—	—
Ca. auris	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
CBS16214	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
Ca. auris	EUCAST versus CLSI	****	<0.0001	****	<0.0001	****	<0.0001	—	—	**	0.0089
DSM105988	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
Ca. auris	EUCAST versus CLSI	****	<0.0001	****	<0.0001	****	<0.0001	—	—	**	0.0022
DSM105990	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	ns	>0.9999	—	—
Ca. auris	EUCAST versus CLSI	****	<0.0001	****	<0.0001	****	<0.0001	—	—	**	0.0089
DSM105992	EUCAST versus SYO	****	<0.0001	—	—	ns	0.1005	—	—	—	—
	CLSI versus SYO	ns	>0.9999	—	—	****	<0.0001	****	<0.0001	—	—
Ca. auris	EUCAST versus CLSI	****	<0.0001	**	0.0038	****	<0.0001	—	—	ns	0.8868
DSM21092	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
C. blankii	EUCAST versus CLSI	****	<0.0001	ns	>0.9999	ns	>0.9999	—	—	**	0.002
NCPF8710	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	ns	>0.9999	—	—	****	<0.0001	****	<0.0001	—	—
C. dubliniensis	EUCAST versus CLSI	**	0.0054	ns	0.9639	ns	0.1005	—	—	ns	0.1962
DSM28723	EUCAST versus SYO	ns	>0.9999	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	**	0.0054	—	—	****	<0.0001	ns	0.7292	—	—
C. duobushaemulonii	EUCAST versus CLSI	****	<0.0001	****	<0.0001	****	<0.0001	—	—	ns	>0.9999
DSM105996	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
C. glabrata	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
ATCC2001	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	**	0.0038	—	—
C. glabrata	EUCAST versus CLSI	****	<0.0001	ns	>0.9999	ns	>0.9999	—	—	ns	0.8868
CBS15716	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
C. guilliermondii	EUCAST versus CLSI	ns	>0.9999	**	0.0038	ns	>0.9999	—	—	****	<0.0001
ATCC7350	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
C. haemulonii	EUCAST versus CLSI	****	<0.0001	ns	0.5034	ns	>0.9999	—	—	ns	0.3097
DSM105998	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
C. krusei	EUCAST versus CLSI	ns	>0.9999	****	<0.0001	****	<0.0001	—	—	ns	0.3097
ATCC6258	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	ns	>0.9999	ns	>0.9999	—	—
Cl. lusitaniae	EUCAST versus CLSI	ns	>0.9999	****	<0.0001	**	0.0066	—	—	ns	0.9615
DSM70102	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
C. metapsilosis	EUCAST versus CLSI	ns	>0.9999	ns	0.8632	****	<0.0001	—	—	****	<0.0001
NCPF8790	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	ns	0.1005	****	<0.0001	—	—

Continued

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Table 4. *Continued*

Strains	Method/drug	FLC		IBF		5-FC		CAS		RZF	
		Adjusted *P* value		Adjusted *P* value		Adjusted *P* value		Adjusted *P* value		Adjusted *P* value	
C. orthopsilosis DSM24508	EUCAST versus CLSI	ns	>0.9999	ns	0.2338	****	<0.0001	—	—	ns	>0.9999
	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—
C. parapsilosis ATCC22019	EUCAST versus CLSI	ns	>0.9999	ns	0.5034	****	<0.0001	—	—	****	<0.0001
	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	ns	>0.9999	—	—
C. parapsilosis CBS1954	EUCAST versus CLSI	****	<0.0001	ns	>0.9999	****	<0.0001	—	—	****	<0.0001
	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	ns	>0.9999	****	<0.0001	—	—
C. tropicalis ATCC750	EUCAST versus CLSI	****	<0.0001	ns	>0.9999	ns	0.1005	—	—	ns	0.8868
	EUCAST versus SYO	****	<0.0001	—	—	****	<0.0001	—	—	—	—
	CLSI versus SYO	****	<0.0001	—	—	****	<0.0001	****	<0.0001	—	—

Pairwise comparisons were conducted using a two-way repeated-measures ANOVA followed by Tukey's post hoc test for normally distributed data or the Friedman test with Wilcoxon post hoc comparisons (Bonferroni corrected) for non-normally distributed data.
, 0.002–0.0089; **, <0.0001; —, not applicable; ns, not significant.

insights into their performance when using the standard reference methods and the commercial SYO assay.

The similarities and differences between the BMD reference methods have been extensively discussed in the literature.[6,9,26,27] Concordance rates between these two methods vary depending on the antifungal agent and fungal species tested. However, the EA for MIC detection typically ranges from 85% to 95%, demonstrating a high level of concordance. In this study, the EA between two methods reached 95%, consistent with these previously reported findings. The largest significant discordance between the reference methods was observed for ibrexafungerp, a novel triterpenoid antifungal that interferes with the fungal cell wall synthesis through inhibition of glucan synthase,[28] showing an EA of 82%. One study has compared CLSI and EUCAST methodologies using ibrexafungerp, the authors reported an EA of 99%.[29] However that study assessed the activity of ibrexafungerp against only the five most common *Candida* species. The lower EA observed for ibrexafungerp likely reflects intrinsic differences in testing methodologies and the challenges of establishing consistent MIC endpoints for this novel antifungal. A key factor contributing to these discrepancies may be variations in glucose concentrations and inoculum size across testing methods, both of which can significantly influence fungal metabolism and, consequently, drug susceptibility. Similar challenges have been reported for other antifungals affected by metabolic conditions.[30] More comprehensive studies are required to thoroughly understand the performance of ibrexafungerp across diverse yeast species through BMD reference methods. Studies evaluating ibrexafungerp activity via EUCAST typically report MICs of ≤2 mg/L for *Candida* species.[31,32] In our study, most strains similarly exhibited MICs of ≤2 mg/L, except for *C. duobushaemulonii* DSM105996, which had an MIC of 4 mg/L. When ibrexafungerp is evaluated using the CLSI method, MIC values were generally lower, as has also been observed in a study involving fluconazole-susceptible and -resistant *Candida* isolates (MICs ≤ 0.05 mg/L).[33] In addition, Quindós *et al*.,[34] observed the same tendency of higher MIC values for 11 *Candida* species using the EUCAST method; in that study the lowest ibrexafungerp MICs were observed for *C. albicans* (geometric MIC 0.062 mg/L, MIC range 0.016–0.5 mg/L) and the highest ibrexafungerp MICs were observed for *C. tropicalis* (geometric MIC 0.517 mg/L, MIC range 0.06–≥8 mg/L).

When analysing the MICs obtained for the echinocandins, rezafungin, a next generation echinocandin with a modified choline moiety at the cyclic echinocandin core, demonstrated the highest *in vitro* antifungal activity within the group. However, its activity was closely followed by anidulafungin and micafungin, indicating comparable efficacy among these agents. Caspofungin ranked fourth in terms of antifungal activity, with GM values two to three times higher than those of the other echinocandins. A multi-continent study conducted by Carvalhaes *et al*.,[35] on 1679 isolates (yeast and moulds) using the CLSI method found that most common *Candida* species had MIC values of ≤0.05 mg/L, except for *C. parapsilosis*, which exhibited MICs of 1–2 mg/L; results consistent with those observed in our study. Regarding manogepix, which targets the conserved fungal glycosylphosphatidylinositol-anchored cell wall transfer protein 1 (Gwt1),[36] the MICs obtained in this study were the lowest observed (GM: 0.01 mg/L). This reflects its broad spectrum of activity against major fungal pathogens, including *Candida* species (except for *C. krusei*). Previous studies have similarly reported low MICs, with values around 0.06 mg/L for most *Candida* species, including *Ca. auris*.[37,38]

When comparing the performance of BMD reference methods to the commercial SYO method, studies often emphasize both the strengths and limitations of each approach. For instance, Altinbaş *et al*., conducted a comparative study between the CLSI and SYO methods for five conventional antifungal agents against 129 *Candida* isolates, demonstrating high levels of agreement. They reported over 90% EA (±2-fold dilutions) for most antifungal agents, including an EA of 98% for amphotericin B

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and 90% for micafungin.[39] In our study, we observed 100% EA for both drugs. However, it is important to note that a concordance issue between the SYO method and reference methods for *Ca. auris* has recently been reported, particularly in the context of overestimating resistance to amphotericin B.[40] We observed a similar issue when using the VITEK®2 system.[6] Despite the small number of *Ca. auris* strains (five) in our study, this problem was observed with the strain *Ca. auris* DSM105990. Additionally, higher MICs for amphotericin B were consistently observed in *Ca. auris* strains when using the SYO method. This discrepancy may be attributed to the fact that SYO was optimized primarily for common *Candida* species, meaning that testing emerging species such as *Ca. auris* may pose challenges due to its unique biological characteristics, including its growth rate, growth pattern and metabolic status.[40] Recent studies have highlighted significant discrepancies in amphotericin B MIC values depending on the AFST method used.[6,11] Siopi *et al*. evaluated amphotericin B susceptibility in 65 *Ca. auris* clinical isolates using SYO and the CLSI reference method, reporting markedly lower MIC values with CLSI compared with SYO. The EA (within ±1 2-fold dilution) between SYO and CLSI was only 29%, and the CA, based on the CDC breakpoint (MIC ≥ 2 mg/L), was just 11%, with 89% major errors (MaE). This suggests that SYO significantly overestimates amphotericin B resistance in *Ca. auris* isolates when following the manufacturer's instructions and using the CDC breakpoint. Interestingly, they demonstrated that modifying the interpretative criteria improved the concordance. When applying the SYO WT upper limit MIC of 8 mg/L, major errors were eliminated (0% MaE). Similarly, defining MIC endpoints based on growth inhibition rather than colorimetric changes (MIC-1 at 75% inhibition and MIC-2 at 50% inhibition) increased CA to 88% (12% MaE) and 97% (3% MaE), respectively.[40] With only a suggested susceptible breakpoint and no intermediate category, the inherent margin of error of ±1 2-fold dilution can lead to EA while still causing categorical disagreement, particularly for isolates with MIC values of 1 (susceptible) and 2 mg/L (resistant).[41]

On the other hand, a high rate of discrepancy was identified between the SYO and CLSI methods for 5-flucytosine and caspofungin. Notably, the low correlation between these two methods for caspofungin has been observed previously.[42] Interestingly, a higher percentage of agreement was observed between the SYO and EUCAST (91%) methods compared with SYO and CLSI (89%). This is primarily due to the absence of caspofungin testing in the EUCAST method. Additionally, for 5-flucytosine, the MICs obtained with EUCAST were slightly higher than those from CLSI, which increased the percentage of agreement between SYO and EUCAST. The lower EA (64%) for 5-flucytosine, is in line with prior reports highlighting greater variability in MIC results, particularly due to differences in reading endpoints. The presence of trailing growth and variations in metabolic activity across isolates can complicate MIC determination, leading to discrepancies between methods. Additionally, 5-flucytosine is known for its rapid *in vitro* development of resistance, which may contribute to method-dependent differences.[43]

We recommend that users of the EUCAST method for determining MICs ensure that their plate reader performs multiple readings within each well. Relying solely on a single OD reading can lead to inaccurate MIC values. In our observations, some MICs appeared artificially higher when only one OD value was considered. To improve accuracy, we suggest performing multiple readings per well and visually (e.g. use of an inverted mirror) verifying the results by checking for correlation with the plate reader's determination. This dual approach can help avoid misinterpretation of MIC values.

Despite the generally high concordance observed among the methods, discrepancies emerge when clinical breakpoints or epidemiological cut-off values are applied for antifungal susceptibility interpretation. These discordances are predominantly related to the SYO method (CLSI breakpoints). Notably, in the case of posaconazole and voriconazole against *C. glabrata*, the SYO method categorizes the strains as non-WT, whereas the other methods do not. Conversely, for caspofungin, the opposite pattern is observed: strains identified as non-WT/resistant by CLSI are not detected by the SYO method. Interlaboratory variability of caspofungin MICs has been reported, which is why it is recommended to use other echinocandins to confirm resistance.[44] In this study, when micafungin and anidulafungin were evaluated, no resistance was observed in strains that were categorized as caspofungin-resistant. However, it is important to note that the MICs for these caspofungin-resistant strains were close to the established breakpoint. For antifungal agents such as itraconazole, amphotericin B, anidulafungin and micafungin, all three methods demonstrate complete categorical concordance in cases where cut-off points are available. Similar findings have been described elsewhere.[42,45,46]

One limitation of this study is its single-centre design, which may restrict the generalizability of the results to broader settings. Additionally, the small sample size, with typically only one strain representing each species, further constrains the applicability of the findings and highlights the need for broader, multicentre studies to validate these results comprehensively. Unfortunately, we were unable to include all strains from the CDC's AR-Bank, as these strains are only available for purchase by researchers based in the United States. Despite these constraints, obtaining performance data from various testing methods on strains widely used as reference standards is of high relevance, especially in the context of evaluating novel antifungal agents. Given the international importance of both the CLSI and EUCAST methods in clinical testing and antifungal resistance surveillance, it is crucial to continue efforts towards harmonizing these methods for testing not only novel antifungals but also established agents. Such harmonization will help ensure more reliable and consistent results across laboratories worldwide. Establishing a new mycology laboratory enabled us to integrate both identification and AFST methods within our institution. We encourage researchers worldwide who are setting up their laboratories or methodologies to leverage the data obtained, which can serve both to validate internal protocols and to present and compare findings with the scientific community.

This study highlights the perfect concordance achieved in the identification of strains using both molecular and proteomic methods. Additionally, highlights the significant concordance between the EUCAST and CLSI methods, demonstrating high EA across widely used antifungal agents such as amphotericin B and anidulafungin, thereby supporting their reliability in clinical testing. The SYO method also proved to be a robust alternative, exhibiting considerable agreement with both EUCAST and CLSI methodologies.

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Nevertheless, despite using reference strains, it is crucial to verify their identification, as strains may arrive contaminated or incorrectly labelled. This was evident in our study, where one reference strain was found to be contaminated with another *Candida* species upon arrival at our institution. Such discrepancies highlight the need for rigorous quality control in fungal identification and susceptibility testing.

Importantly, the emergence of novel antifungal agents, such as ibrexafungerp and manogepix underscores the necessity for continuous evaluation and standardization of AFST methods. These agents offer promising therapeutic options for resistant fungal infections. However, their effective integration into clinical practice requires robust susceptibility testing protocols to ensure accurate and reproducible results. Ongoing surveillance and method validation are critical to address the evolving challenges posed by antifungal resistance and to optimize patient outcomes.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC-AMR* Online.

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