Mycopathologia (2024) 189:64
https://doi.org/10.1007/s11046-024-00861-2

REVIEW

Twenty Years in EUCAST Anti-Fungal Susceptibility Testing: Progress & Remaining Challenges

Maiken Cavling Arendrup · Jesus Guinea · Joseph Meletiadis

Received: 12 March 2024 / Accepted: 15 May 2024 / Published online: 11 July 2024

Abstract Since its inception in 2002, the EUCAST Antifungal Susceptibility Testing Subcommittee (AFST) has developed and refined susceptibility testing methods for yeast, moulds and dermatophytes, and established epidemiological cut-off values and breakpoints for antifungals. For yeast, three challenges have been addressed. Interpretation of trailing growth in fluconazole susceptibility testing, which has been proven without impact on efficacy if below the 50% endpoint. Variability in rezafungin MIC testing due to laboratory conditions, which has been solved by the addition of Tween 20 to the growth medium in E.Def 7.4. And third, interpretation of MICs for rare yeast with no breakpoints, where recommendations have been established for MIC-based clinical advice. For moulds, refinements include the validation of spectrophotometer reading for *A. fumigatus* to facilitate objective MIC determination, and for dermatophytes the establishment of a microdilution method with automated reading and a selective medium to minimise the risk of contaminations. Recent initiatives involve development and validation of agar-based screening assays for detection of potential azole and echinocandin resistance in *A. fumigatus* and *Aspergillus* species, respectively, and of terbinafine resistance in *Trichophyton* species. Moreover, the development of a EUCAST guidance document for molecular resistance testing represents an advancement, particularly for identifying target gene alterations associated with resistance. In summary, EUCAST AFST continues to play a pivotal role in standardizing AFST and facilitating accurate interpretation of susceptibility data for clinical decision-making. Adoption of EUCAST breakpoints for commercial test methods, however, requires thorough validation to ensure concordance with EUCAST reference testing species-specific MIC distributions.

Keywords Susceptibility testing · Microdilution · Antifungal · Yeast · Mould · Dermatophyte

Handling Editor: Lore Vanderbeke.

M. C. Arendrup (✉)
Unit for Mycology, Statens Serum Institut, Building 45/112, Artillerivej 5, 2300 Copenhagen, Denmark
e-mail: maca@ssi.dk

M. C. Arendrup
Department Clinical Microbiology, Rigshospitalet, Copenhagen University, Copenhagen, Denmark

J. Guinea
Clinical Microbiology and Infectious Diseases, Hospital General Universitario Gregorio Marañón, 28007 Madrid, Spain

J. Guinea
Faculty of Health Sciences-HM Hospitals, Universidad Camilo José Cela, Madrid, Spain

J. Meletiadis
Clinical Microbiology Laboratory, Attikon University Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece

Introduction

Organisation of the EUCAST Antifungal Subcommittee

The EUCAST committee on antimicrobial susceptibility testing, deals with clinical breakpoints (BPs) and technical aspects of phenotypic in vitro susceptibility testing and functions as the breakpoint committee of European Medicines Agency (EMA) & European Centre for Disease Prevention and Control (ECDC). For new agents, BPs are set as part of the licensing process through EMA or national medicines agencies. EUCAST antifungal susceptibility testing subcommittee (AFST) does the above for antifungal agents, including to develop and optimise reference methods, and to propose epidemiological cut off values (ECOFFs) and BPs for new and already licensed antifungal agents. However, EUCAST steering committee (SC) makes the final decision on the proposals from the EUCAST AFST in order to ensure alignment across antibacterial and antifungal testing and interpretation principles. All approved documents are freely available on the www.EUCAST.org website, under the AST of Fungi tab.

The EUCAST AFST subcommittee was formed as an open committee in 2002 and consisted at that time of a chairman, a secretary, a representative from the EUCAST steering committee and interested mycologists from Europe that met annually during ECCMID

Table 1 The organisational history of the EUCAST antifungal susceptibility testing subcommittee steering committee (EUCAST AFST SC)

	Chair	Secretary	Data-coordinator	EUCAST representative	NAC	NAC
2002	JL Rodriguez-Tudela (ES)	P Donnelly (NL)		J Mouton (NL)	Open committee	
2003						
2004					J Bille (CH), D Denning (GB), C Lass-Floerl (AT), M Cuenca-Estrella (ES), MC Arendrup (DK)	
2005						
2006						
2007						
2008						
2009						
2010						
2011	MC Arendrup (DK)	W Hope (GB)			C Lass Floerl (AT)	Cuenca-Estrella (ES)
2012						
2013						
2014		S Howard (GB)	J Meletiadis (GR)			
2015					K Lagrou (BE)	J Guinea (ES)
2016		J Guinea (ES)				P Hamal (CZ)
2017					F Barchiesi (IT)	
2018						M Mares (RO)
2019				G Kahlmeter (SE)	N Friberg (FI)	
2020						S Arikan (TR)
2021					K Muehlethaler (CH)	
2022						
2023				C Giske (SE)		P Lyskova (CZ)

The two letter country ISO codes (ISO 3166 standard) are used for indicating the nationality of the SC members

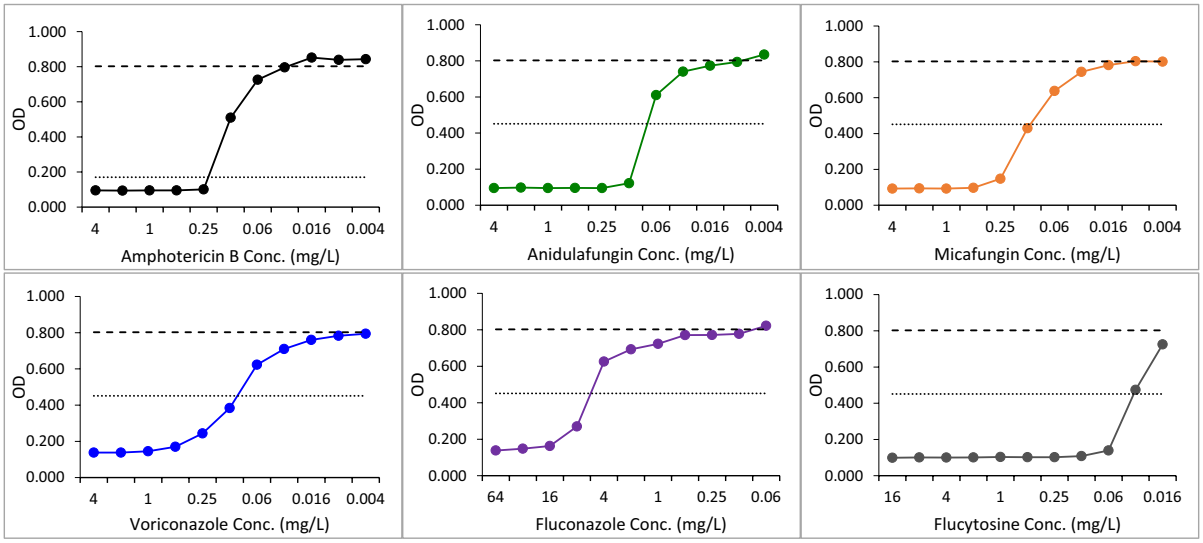

Fig. 1 EUCAST AFST E.def 7.4 MIC testing of a *S. cerevisiae* isolate. Growth curves for left to right show amphotericin B (black), anidulafungin (green) and micafungin (orange) in the upper panel and voriconazole (blue), fluconazole (purple) and flucytosine (grey) in the lower panel. the mean of the growth is indicated as the dashed line and the 90% and 50% growth inhibition cut off lines as dotted lines. MICs are read as the lowest concentration before the growth curve intersects the inhibition cut off line (amphotericin B: 0.25 mg/L, anidulafungin 0.125 mg/L, micafungin 0.125 mg/L, voriconazole 0.125 mg/L, fluconazole 8 mg/L and flucytosine 0.06 mg/L)

conferences (Table 1). In 2004, a steering committee was formed, in 2011 the representation of countries on the steering committee was formalised to 2-year rotating national representatives (new countries given preference) and in 2014 the EUCAST AFST SC was supplemented with a data coordinator. Consequently, the EUCAST AFST SC today has the same format (but fewer members) as the EUCAST AST steering committee. The chair is appointed by the EUCAST SC and the secretary and data coordinator are appointed by the EUCAST AFST Chair and the EUCAST SC in collaboration.

To keep an arm's length between the EUCAST and the industry, the chair, secretary and data coordinator cannot be employees of pharmaceutical companies, industrial advisory board members or engage in discussion on strategic matters. Other commitments involving the industry (lectures, a travel grant, an invitation to present, etc.) can be considered but must be declared in annual yearly EUCAST update of the "Declaration of conflicting interests".

Overview of Developed EUCAST Methods

Yeast Testing

The first reference method for susceptibility testing of yeast was released as a discussion document in 2003 [1]. The test principle was very similar to the already existing reference method from the Clinical Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standard (NCCLS)). However, EUCAST adopted a higher glucose and inoculum concentration to promote faster growth and incubation in microtitre plates with flat-bottomed wells rather than U-shaped, which allowed an objective spectrophotometer MIC reading after 24 h rather than visual reading after 48 h as originally recommended by CLSI. Examples of growth curves are given in Fig. 1. Since the first method was released as a discussion document and a definitive document in 2008, the method has been further refined as summarised in 7.2, 7.3.1, 7.3.2 and 7.4 (Tables 2 and 3). The second version contained additional information concerning solvent for specific agents, shelf-life of plates containing the echinocandins, testing of

Table 2 Overview of EUCAST documents on susceptibility testing methods, resistance screening method, breakpoints and ECOFFs and guidance publications on interpretation of MICs for rare yeast and for molecular resistance detection for yeast, moulds and dermatophytes

Year	Yeast	Mould	Mould screening	*Trichophyton*	yeast BP	Mould BP	ECOFF-BP overview	Molecular resistance detection
2002								
2003	E.Dis 7.1							
2004								
2005								
2006								
2007					FCZ			
2008	E.Def 7.1	E.Def 9.1			VCZ			
2009								
2010					AMB, VCZ, PCZ			
2011						AMB		
2012	E.Def 7.2					VCZ, ITZ, PCZ		
2013					AFG, MFG, FCZ			
2014		E.Def 9.2			ITZ			
2015		E.Def 9.3				ISZ		
2016								
2017	E.Def 7.3.1	E.Def 9.3.1			VCZ, PCZ			
2018			E.Def 10.1					
2019								
2020	E.Def 7.3.2	E.Def 9.3.2		E.Def 11.0	All RDs revised (Revised "I" category)		September 2020	
2021								
2022		E.Def 9.4	E.Def 10.2				January 2022	JAC 2022
2023	E.Def 7.4				Rare yeast guidance		August 2023	

FCZ, fluconazole; VCZ, voriconazole; AMB, amphotericin B; PCZ, posaconazole; ITZ, itraconazole; AFG, anidulafungin; MFG, micafungin; ISZ, isavuconazole; RD, rationale document; JAC, Journal of Antimicrobial Chemotherapy

Table 3 Comparison of key conditions for EUCAST susceptibility test methods for yeast, mould and dermatophytes

	Yeast E.Def 7.4	Mould E.Def 9.4	Derm E.Def 11.0
Glucose conc.	2% (& Tween 20 for RZF*)	2%	2%
Selective medium?	No	No	Yes**
Inoculum size	0.5–2.5 × 10^5 (spec^/counted)	1–2.5 × 10^5 (spec/counted)	1–2.5 × 10^5 (spec/counted)
Plates & reading	Flat & Spec	Flat & Spec/Visual	Flat & Spec
Incubation time and temp	24 h 37 °C (30 °C)	48 (24–72) h 37 °C (30 °C)	5 (− 7) days, 25–28 °C
Endpoint (growth inhibition)	≥ 90% AMB*** ≥ 50% others	"Complete visual" ≥ 90%	≥ 50%

*RZF: rezafungin; **the medium is rendered selective for dermatophytes by supplementation with cycloheximide and chloramphenicol; ^spectrophotometrically; ***AMB: amphotericin B

Cryptococcus and reference MIC ranges for quality control (QC) strains and anidulafungin. The third version introduced the revised definition of the "I" category, from "**I**ntermediate" to "susceptible, **I**ncreased exposure", and a section concerning the preparation and calibration of the spectrophotometer was modified [2]. In addition, MIC targets and ranges for QC strains were removed acknowledging the new specific QC document. During the same time period, CLSI also refined their yeast method allowing MICs to be read after 24 h of incubation [3–7].

Challenges and Solutions

Although this gradual refinement of the method document led to reproducible and easy to read MICs, three challenges were acknowledged. The first was that some isolates are only partially inhibited by fluconazole leading to growth curves that were worryingly close to the 50% inhibition threshold and therefore caused uncertainty regarding whether the isolate could be safely interpreted as fluconazole susceptible. To address this question, we evaluated *C. tropicalis* isolates with different degrees of trailing in *Galleria mellonella* larvae and in mice experiments [8]. Six isolates where chosen that displayed upon repeated testing either a robust phenotype of low trailing, or of weak to modest trailing below or around the 50% inhibition line, or of strong trailing growth consistently above the 50% inhibition line yet were still supressed, and finally a classical resistant isolate with MIC 16 mg/L and no suppression of growth in the $\leq$4 mg/L concentration range. In the *Galleria mellonella* larvae model, a stepwise increase in mortality was observed in parallel with increased fluconazole trailing in vitro for all three fluconazole doses used (1–20 mg/kg). But in both an immunocompetent and a neutropenic mouse model of haematogenous candidiasis, only *C. tropicalis* isolates with consistent trailing above the 50% growth inhibition endpoint and the frank resistant *C. tropicalis* isolate responded less well to therapy [8]. Consequently, EUCAST recommends to ignore trailing growth as long as it is below the 50% growth inhibition cut-off line.

Another challenge associated with the EUCAST microdilution testing, is that some drugs are associated with unacceptable inter-laboratory MIC variation. This has been seen particularly for agents that are highly active at a mg/L basis, likely because even small variation or losses of drug will inevitably impact the available amount of free drug in the wells around the (low) MIC. Robust MIC testing is key, both for setting breakpoints and for subsequent correct interpretation of MICs. This is ensured through strict criteria for ECOFF and breakpoint setting. A first step in EUCAST ECOFF setting is to collect MIC distributions and evaluate whether they qualify for aggregation. The criteria for this evaluation require at least five MIC distributions that are non-clonal (outbreak isolates are not accepted), non-truncated and mono-modal with most MICs usually covering 3–5 twofold dilutions and derived from different laboratories. Furthermore, to qualify for aggregation each distribution must consist of at least 15 isolates and each distribution must have a mode that is within $\pm$1 dilution from the most common mode across all available MIC distributions. Finally, a total of at least 100 isolates in the aggregated distribution is required [9]. During the process of collecting rezafungin MIC distributions against *Candida* spp., unacceptable variability was noted especially for clinical isolates and reference strains of the lowest MIC species most often *C. albicans* [10]. This variation was found to be related to the choice of plastic trays used for microdilution preparation [11]. EUCAST cannot mandate the use of specific brands of plates, and even if that was done, such plates are not developed and validated specifically for the use for AFST and therefore not protected against or validated for future changes in composition and production that might affect performance. Hence, it was necessary to find a method for avoiding loss of free drug due to sticking to plastics in tubes, reservoirs and microtitre trays. Inspired by similar challenges related to antibacterial testing of glycopeptides, where surfactant (tween, polysorbate) was found to abrogate binding and loss compound in plastics, Tween 20 supplementation of the growth medium was evaluated with promising results [12, 13]. This approach was subsequently validated in a multicentre study including both wild-type isolates as well as *fks* mutant isolates harbouring weak and strong mutations and shown to lower the variation of GM-MICs across the centres for *C. albicans* from 4.8 to 2.6 fold and lead to data set complying with the rule of modes being within $\pm$1 dilution from the most common mode [14]. This method is now included in the newest version of the method document E.Def 7.4. One important caveat,

that clinical laboratories have to pay attention to, is the consequent notable difference in rezafungin MICs obtained by the EUCAST method and CLSI method, respectively. The MIC_{50} for *C. albicans* was 0.001 mg/L with Tween 20 supplementation, whereas the MIC_{50} with CLSI testing is 0.03 mg/L and thus 5 two-fold dilutions higher than the MIC_{50} with the modified EUCAST method [15]. In Fig. 2, the EUCAST and CLSI rezafungin breakpoints for *Candida* spp. are summarised and difference visualised between MICs obtained with the two methods for *C. albicans*. Obviously, it is crucial to adopt the correct breakpoints for MIC interpretation due to the 1–5 fold difference in MICs between the methods. Adopting the EUCAST breakpoints for CLSI MICs will categorise virtually the entire wild-type MIC population as resistant, whereas adopting CLSI breakpoints for EUCAST MICs will allow more than a 5 two-fold dilution MIC increase without resulting in a *C. albicans* isolate being classified as resistant. Due to this difference any commercial test must be aligned with and interpreted by either EUCAST or CLSI methods and breakpoints, and laboratories must be aware of choosing the right breakpoints for the method they use. Moreover the early publications on EUCAST MICs obtained by E.Def 7.3 are not valid for interpretation with the final breakpoints based on the E.Def 7.4 method.

A third challenge has been setting clinical breakpoints for rare yeast. Setting breakpoints requires species specific clinical outcome data because the virulence of the different *Candida* species varies notably [16]. However, species specific clinical outcome data for infections with rare yeast or moulds will not be available in the foreseeable future. Recently, a pragmatic classification for rare yeast, which relies on two important assumptions was proposed [17]. First, when isolates are genetically related, pathogenicity and intrinsic susceptibility patterns are assumed to be similar. Second, even if species are not phylogenetically related, the rare yeasts will likely respond to therapy provided the MIC is comparable to values observed in wild-type isolates of more prevalent susceptible species. This second assumption relies on the assumption that rare yeasts are likely less pathogenic than the common *Candida* spp. as they would otherwise not be rare. In house MIC distributions including more than 4000 isolates were inspected and validated against published EUCAST MIC data. In addition, treatment recommendations available in the current guidelines were taken into consideration [18, 19]. This approach has now been accepted by EUCAST with the recommendation to avoid interpretation of especially S and I, as use of these abbreviations may mislead the reader to believe the classification is based on the same extensive amount of data that underlies EUCAST clinical breakpoints (Table 4).

Mould Testing

The method developed for moulds rely on the same principle as the yeast method although with the following modifications, (1) a slightly lower and more narrow inoculum size, (2) an incubation duration

a)

Species	EUCAST Tween E.Def 7.4 S/R[a] (mg/L)	CLSI S/R[a] (mg/L)	Number of 2-fold dilution difference
C. albicans	0.008/0.008	≤0.25/-[b]	5
C. dubliniensis	0.016/0.016	≤0.125-	3
C. glabrata	0.016/0.016	≤0.5/-	5
C. krusei	0.03/0.03	≤0.25/-	3
C. parapsilosis	4/4	≤2/-	1
C. tropicalis	0.03/0.03	≤0.25/-	3
C. auris	IE	≤0.5/-	-

[a] S/R breakpoints are indicated a S ≤ x / R >x.

[b] CLSI has not established resistance breakpoints for *C. auris*.

b)

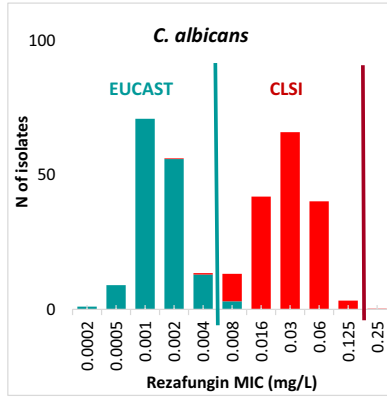

Fig. 2 EUCAST and CLSI breakpoints for rezafungin for the five most common *Candida* species and *C. auris* (**a**) and a histogram comparing EUCAST E.Def 7.4 and CLSI M-27 document MIC distributions for *C. albicans* retrieved from Arendrup et al. [14] and Carvalhaes et al. [15]. The vertical lines indicate the EUCAST (turquoise) and CLSI (red) breakpoints (**b**)

Table 4 A pragmatic approach to MIC interpretation for rare yeast species without EUCAST breakpoints

Recommendation regarding treatment	Amphotericin B	Anidulafungin	Fluconazole	Voriconazole
Can be used when…	Confirmed MIC ≤ 1 mg/L: *Candida* species Rare yeasts (except those below)	Confirmed MIC ≤ 0.06 mg/L: *C. dubliniensis* *C. inconspicua* *C. nivariensis* *C. norvegensis* *C. pelliculosa* *C. utilis* *L. elongisporus* *P. kluyveri* Repeat MIC ≤ 0.125 mg/L or perform *fks* sequencing *C. intermedia* *C. lusitaniae* *C. palmioleophila* *C. kefyr*	Confirmed MIC ≤ 2 mg/L: *C. intermedia* *C. kefyr* [MIC ≤ 1] *C. lusitaniae* *C. metapsilosis* *C. orthopsilosis* *C. utilis* *L. elongisporus*	Confirmed MIC ≤ 0.03 mg/L: *C. intermedia* *C. kefyr* *C. lusitaniae* *C. metapsilosis* *C. orthopsilosis* *L. elongisporus*
Can be considered for use in the following situations, provided MICs are within the indicated ranges: Not severe infection/ Elevated dose/ Oral consolidation/ No better options		Confirmed MIC 0.125–0.5 mg/L: →consider use in some situations (for ex. less severe infections, when no better option is available) *C. lipolytica* *C. magnoliae* *C. metapsilosis* *C. orthopsilosis* *C. pararugosa* *S. cerevisiae* *A. adeninivorans*	Confirmed MIC 2–16 mg/L: →consider use in some situations (increased dosage and less severe infections) *C. fermentati* *C. nivariensis* *C. pararugosa* *C. pelliculosa* *C. guilliermondii* [MIC ≤ 16] *C. palmioleophila* *C. bovina* *T. dermatis* (1st line Alt) *Cr. neoformans* (2nd line) *S. cerevisiae* *T. asahii* (1st line Alt)	Confirmed MIC 0.06–0.125 mg/L: →consider use in some situations (TDM confirmed sufficient exposure, less severe infections or when no better option is available) *C. fermentati* *C. guilliermondii* *C. lipolytica* *C. nivariensis* *C. palmioleophila* *C. pelliculosa* *C. utilis* *S. cerevisiae* *Cr. neoformans* [MIC ≤ 0.5] *T. dermatis* (1st line)
Consider alternative therapy	Confirmed MIC > 1 mg/L: Any isolate →regard resistant *C. lusitaniae* [MIC > 0.5] *Trichosporon* spp. (2nd line)	Repeat MIC 0.5–1 mg/L No evidence that allows recommendation *C. fermentati* *C. guilliermondii* Repeat MIC ≥ 1 mg/L: →regard resistant *Cryptococcus* *Trichosporon, Magnusiomyces, Geotrichum and Rhodotorula* (Against due to intrinsic resistance)	Confirmed MIC > 16 mg/L →regard resistant *C. inconspicua* *C. lipolytica* *C. magnoliae* *C. palmeoliophila* *C. norvegensis* *P. kluyveri* *G. candidum* *R. mucilaginosa* *M. capitatus* *M. clavatus* *A. adeninivorans*	Confirmed MIC 0.25–1 mg/L: No evidence that allows recommendations *C. inconspicua* *C. norvegensis* *P. kluyveri* *M. capitatus* (1st line Alt) *G. candidum* (1st line Alt) Confirmed MIC testing ≥ 2 mg/L →Regard as resistant *A. adeninivorans* *R. mucilaginosa* (Against)

Isolates with MICs above the indicated species-specific values (and thus non-wildtype) should be regarded resistant [17]

Adapted from Astvad et al. [17]

Text in parentheses indicate clinical guideline recommendations (1st line alt: 1st line alternative, 2nd line: use as 2nd line treatment option, Against: recommendation against use). Text in brackets e.g. [MIC ≤ 16] indicates a specific cut off (in mg/L) for that particular species, when it deviates slightly from the group it is placed in

dependent on the organism, typically 24 h for Mucorales, 48 h for *Aspergillus* and most other moulds but allowing 72 h if growth is insufficient (Table 3) [20, 21]. Furthermore, visual reading was adopted because of the uneven growth moulds display in fluid medium and a concern that a single point spectrophotometric reading would fail to capture growth if situated outside the centre of the well. However, visual reading is subjective, time consuming and does not provide any data for later inspection. Consequently, visual and spectrophotometric reading of *A. fumigatus* was compared in three studies including both wild-type and non-wild-type isolates with azole resistance confirming a very high agreement between the two endpoints as well as between susceptibility classification of the isolates [22–24]. It is likely that other species can also be read spectrophotometrically, but the background optical density (OD) level of inoculated but growth inhibited wells of especially *A. niger* but also *A. flavus* and *A. terreus* is higher than for *A. fumigatus* and too close to the 90% inhibition endpoint used for *A. fumigatus* (Fig. 3). This will inevitably lead to random variation in MIC determination and has to be addressed before spectrophotometric reading can be recommended, although visual inspection of the growth curves for these species suggests a solution (higher cut off line) would be possible.

Dermatophyte Testing

With the increasing number of reports documenting a rapid increase of terbinafine resistance in dermatophytes in India and subsequently in other countries and continents due to spread with travellers and immigration and de novo development, a EUCAST susceptibility test method for dermatophytes became of high importance [25–29]. Dermatophytes grow slowly compared to yeast and moulds and are typically cultured at lower temperatures than fungi causing invasive infections

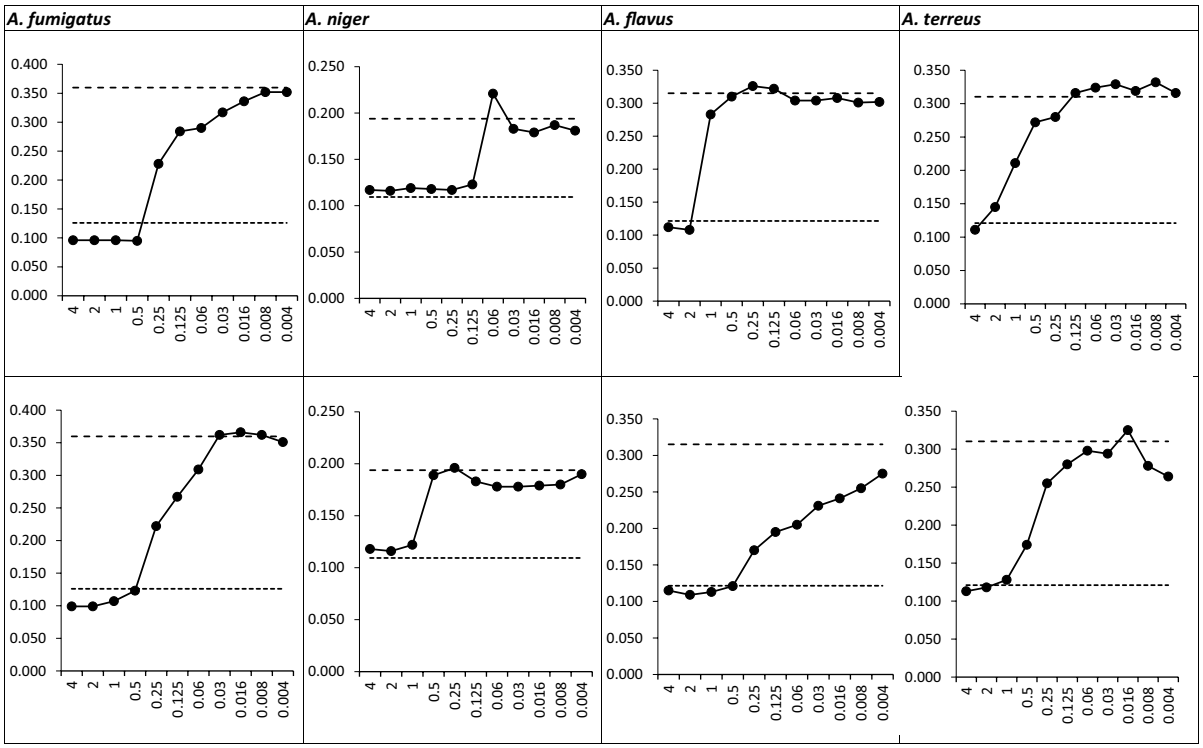

Fig. 3 Susceptibility testing growth curve for amphotericin B (upper panel) and voriconazole (lower panel) against *A. fumigatus*, *A. niger*, *A. flavus* and *A. terreus*. The Y-axis displays the OD value, the X-axis the drug concentration (mg/L), the stippled line the mean of eight positive growth controls and the dotted line the 90% growth inhibition endpoint compared to the background OD of uninoculated medium containing negative control wells

because of their natural site of infection being the cooler nail and skin. Consequently, the microdilution method was adjusted to a lower incubation temperature (25–28 °C) and a prolonged incubation time of 5 (− 7) days. Moreover, to avoid contamination with other fungi, the growth medium was supplemented with cycloheximide and chloramphenicol as is typically used in agars for dermatophyte cultures. The performance of the proposed method was confirmed in a multicentre study using a blinded set of molecularly confirmed terbinafine susceptible and resistant isolates of *T. interdigitale* and *T. indotineae* and the method document E.Def 11.0 released [20, 30].

User-Friendly Resistance Screening Agar Methods

Susceptibility testing with reference microdilution methods are not feasible as a routine analysis in all diagnostic laboratories and though reference laboratories may offer testing, referral of isolates implies a delay before the susceptibility data is available. Therefore, EUCAST developed screening assays for azole resistance in *A. fumigatus* (E.Def 10.1) and echinocandin resistance in *Aspergillus* spp. (E.Def 10.2, Table 2), an initiative that was originally inspired by the studies by Verweij et al. [31–34]. A similar four well agar screening method for detection of terbinafine and itraconazole resistance in *Trichophyton* species is currently validated in single and multicentre studies with promising results [35]. These methods are easy to perform and can, if read correctly, identify isolates that potentially carry resistance mechanisms and for which full MIC testing is important. Of note, the endpoints for the methods differ by drug class as they do for microdilution testing of moulds (MEC versus MIC). For azoles and terbinafine a no growth endpoint at the relevant agar drug concentrations is required for susceptibility classification, whereas for the echinocandin agar screening, an *Aspergillus* is regarded susceptible if an aberrant colony morphology is observed (disk like morphology rather than a colony with a circumference of radiating hyphae similar to the uninhibited growth control, Fig. 4).

Molecular Resistance Testing

One of the recent initiatives, has been to develop a EUCAST guidance document for molecular resistance testing of fungi [36]. Compared to the bacterial field, advantages in molecular mycology have been delayed, in part because of the increased complexity related to the larger fungal genome and many species being diploid. Antifungal resistance can be solely due to target gene alterations, which allows detection of resistance by gene sequencing or direct detection of specific target gene mutations by PCR [37]. This is the case in the majority of cases of azole resistance in *Aspergillus*, terbinafine resistance in dermatophytes, and echinocandin resistance in *Candida*. So far, a relatively limited number of different alterations have caused the majority of terbinafine resistant *Trichophyton* and azole resistant *A. fumigatus* cases, and commercial kits have been developed for their direct detection [36]. For echinocandin resistance in *Candida* it is more complex because resistance mutations can occur in two hotspots of the *fks1* target gene and in *C. glabrata* also of the *fks2* gene, and because the magnitude of the MIC elevation depend on which codon is involved and which amino acid is replacing the wild-type one [38]. Consequently, commercial molecular assays for echinocandin resistance in *Candida* are not yet available. For azole resistance in *Candida*, target gene alterations, efflux pumps and upregulation of target gene expression often play in concert, rendering molecular resistance detection more complicated. Nevertheless, with the emergence of fluconazole resistant *C. parapsilosis* harbouring a specific Y132F alteration in a number of countries in southern Europe and elsewhere, *erg11* sequencing or direct detection of the underlying mutation by PCR has become increasingly helpful and relevant [39–44].

Challenges in Adopting EUCAST Clinical Breakpoints for Commercial Test Methods

Adoption of EUCAST BPs will only result in a correct susceptibility classification for MICs obtained by a commercial test, if the test in question yields MICs that mirror those obtained by EUCAST reference testing, which is not always the case [45]. Before implementing a susceptibility test in the clinical routine,

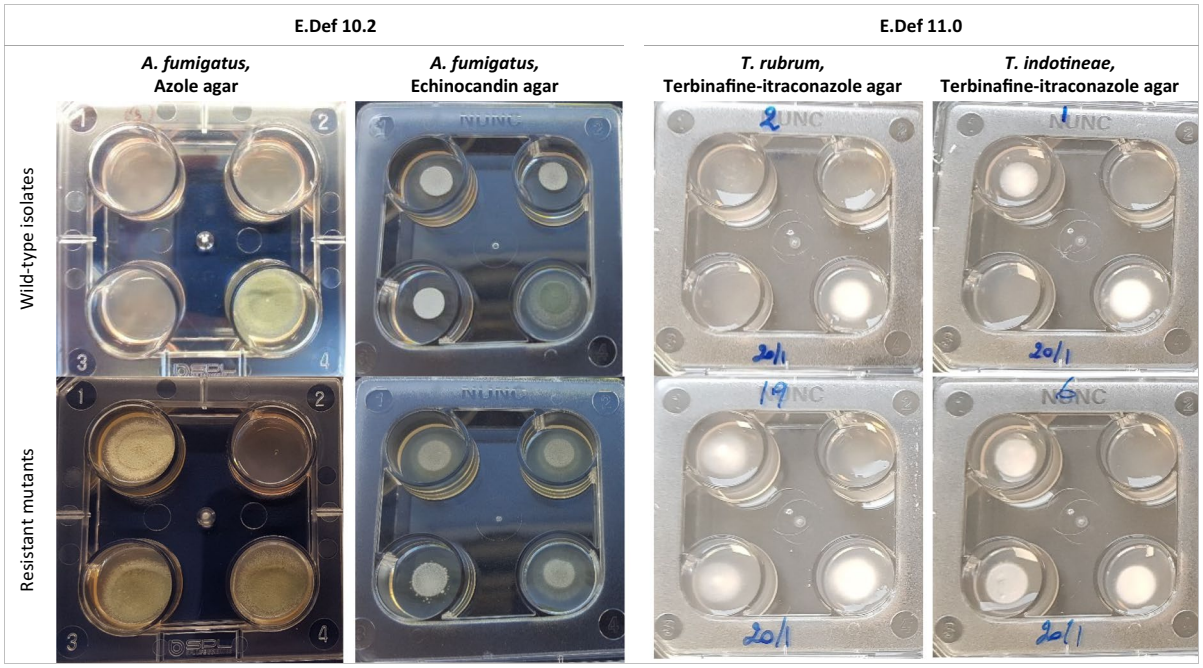

Fig. 4 EUCAST Agar screening assays for detection of presumptive resistance in *Aspergillus* and *Trichophyton* species. Upper row displays wild-type isolates and the lower row non-wild-type isolates with molecularly confirmed resistance mutations[a]. Well 4 is the drug free control on all plates. Well 1 to 3 contain the following agents: Azole agars: itraconazole, voriconazole and posaconazole, echinocandin agars: caspofungin, anidulafungin and micafungin, and dermatophyte agars: terbinafine low, itraconazole and terbinafine high concentration. [a]Lower row from left to right: non-wild-type isolates containing the following target protein alterations: *A. fumigatus* Cyp51a G54W; *A. fumigatus* Fks1 S678P, *T. rubrum* SQLE F397L, *T. indotineae* SQLE L393F

EUCAST AFST advices first to test the EUCAST fungal QC strains 10 times each and to check if the modal MIC is on the target and the MICs within the range. Random variation is permissible (maximum 1 of 10 MIC values outside reference range), but systematic deviation (modal MIC systematically to one side of the target) is not. If this test shows a good performance, the second step is to test 10 clinical isolates of each of the common species (*C. albicans*, *C. glabrata*, *C. krusei, C. parapsilosis* and *C. tropicalis*), which together cover the relevant antifungal ranges for most antifungal agents. Again, the modal MICs should be within ± 1 dilution of the modal MICs for each of the drug-bug combinations for each fungal species found in the EUCAST rationale documents. If they are, the inhouse laboratory performance is suitable for adopting EUCAST breakpoints. If not, there is a high likelihood that the susceptibility test will lead to misclassifications of isolates if EUCAST breakpoints are used for interpretation. Finally, if available, it is advised to consider to test some strains with characterised resistance mutations for a verification that such isolates are correctly identified.

Conclusion

As described above, susceptibility methods have been developed and refined for yeast, mould and dermatophyte testing over the past 20 years, and in the same period EUCAST clinical breakpoints have been developed for the common species, considering dosing regimens used, MIC distributions and ECOFFs [9], pharmacokinetic/pharmacodynamic relationships and species specific MIC dependent clinical outcome relationships, preferably including both wild-type and mutant isolates. Moreover, resistance screening agar methods have been developed for rapid detection of some more common or emerging resistant drug bug combinations. What would facilitate implementation

of these methods in clinical routine laboratories is the commercial availability of ready to use EUCAST trays. Hopefully this will come in the near future.

Acknowledgements This manuscript was prepared without any finding

Author Contributions This manuscript is based upon the Drouhet and the Meet the expert (ME11) lectures held by Maiken Cavling Arendrup at the TIMM conference in Athens 2023. The underlying EUCAST work on method development, ECOFF and BP setting and refinements have been a strong collaboration among the authors. The first draft of the manuscript was written by Maiken Cavling Arendrup and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Declarations

Conflict of interest Outside the current work the author declares the following: M.C.A. has, over the past 5 years, received research grants/contract work (paid to the SSI) from Cidara, F2G, Gilead, and Scynexis, and speaker honoraria (personal fee) from Astellas, Chiesi, Gilead and F2G. She is the current chairman of the EUCAST-AFST. J.M. has received research grants/contract work (paid to the University of Athens) from Gilead, Pfizer and Astelas.

References

1. Cuenca-Estrella M, Moore CB, Barchiesi F, Bille J, Chryssanthou E, Denning DW, et al. Multicenter evaluation of the reproducibility of the proposed antifungal susceptibility testing method for fermentative yeasts of the antifungal susceptibility testing subcommittee of the european committee on antimicrobial susceptibility testing (AFST-EU. Clin Microbiol Infect. 2003;9:467–74.
2. Arendrup MC, Friberg N, Mares M, Kahlmeter G, Meletiadis J, Guinea J, et al. How to interpret MICs of antifungal compounds according to the revised clinical breakpoints v. 10.0 European committee on antimicrobial susceptibility testing (EUCAST). Clin Microbiol Infect. 2020;26:1464–72.
3. Clinical Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. 4th ed. CLSI standard M27. Clinical and Laboratory Standards Institute, Wayne, PA; 2017.
4. Ostrosky-Zeichner L, Rex JH, Pfaller MA, Diekema DJ, Alexander BD, Andes D, et al. Rationale for reading fluconazole MICs at 24 hours rather than 48 hours when testing *Candida* spp. by the CLSI M27–A2 standard method. Antimicrob Agents Chemother. 2008;52:4175–7.
5. Espinel-Ingroff A, Canton E, Peman J, Rinaldi MG, Fothergill AW. Comparison of 24-hour and 48-hour voriconazole MICs as determined by the clinical and laboratory standards institute broth microdilution method (M27–A3 document) in three laboratories: results obtained with 2,162 clinical isolates of *Candida* spp. and othe. J Clin Microbiol. 2009;47:2766–71.
6. Pfaller MA, Andes D, Diekema DJ, Espinel-Ingroff A, Sheehan D. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: time for harmonization of CLSI and EUCAST broth microdilution methods. Drug Resist Updat. 2010;13:180–95.
7. Pfaller MA, Boyken LB, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, et al. Validation of 24-hour posaconazole and voriconazole MIC readings versus the CLSI 48-hour broth microdilution reference method: application of epidemiological cutoff values to results from a global *Candida* antifungal surveillance program. J Clin Microbiol. 2011;49:1274–9.
8. Astvad KMT, Sanglard D, Delarze E, Hare RK, Arendrup MC. Implications of the EUCAST trailing phenomenon in *Candida tropicalis* for the in vivo susceptibility in invertebrate and murine models. Antimicrob Agents Chemother. 2018;62:1–10. https://doi.org/10.1128/AAC.01624-18.
9. European Committee on Antimicrobial Susceptibility Testing. MIC distributions and epidemiological cut-off value (ECOFF) setting, EUCAST SOP 10.2, 2021
10. Arendrup MC, Meletiadis J, Zaragoza O, Jørgensen KM, Marcos-Zambrano LJ, Kanioura L, et al. Multicentre determination of rezafungin (CD101) susceptibility of *Candida* species by the EUCAST method. Clin Microbiol Infect. 2018;24:1200–4.
11. Hare RK, Gertsen JB, Astvad KMT, Degn KB, Løkke A, Stegger M, et al. In vivo selection of a unique tandem repeat mediated azole resistance mechanism (TR120) in *Aspergillus fumigatus* cyp51A, Denmark. Emerg Infect Dis. 2019;25:577–80.
12. Zuill DE, Almaguer AL, Donatelli J, Arendrup MC, Locke JB. Development and preliminary validation of a modified EUCAST yeast broth microdilution MIC method with tween 20-supplemented medium for rezafungin. J Antimicrob Chemother. 2023;78:1102–10. https://doi.org/10.1093/jac/dkad055.
13. Arhin FF, Sarmiento I, Belley A, McKay GA, Draghi DC, Grover P, et al. Effect of polysorbate 80 on oritavancin binding to plastic surfaces: implications for susceptibility testing. Antimicrob Agents Chemother. 2008;52:1597–603.
14. Arendrup MC, Arikan-Akdagli S, Castanheira M, Guinea J, Locke JB, Meletiadis J, et al. Multicentre validation of a modified EUCAST MIC testing method and development of associated epidemiologic cut-off (ECOFF) values for rezafungin. J Antimicrob Chemother. 2023;78:185–95. https://doi.org/10.1093/jac/dkac373.
15. Carvalhaes CG, Klauer AL, Rhomberg PR, Pfaller MA, Castanheira M. Evaluation of rezafungin provisional CLSI clinical breakpoints and epidemiological cutoff values tested against a worldwide collection of contemporaneous invasive fungal isolates (2019 to 2020). J Clin Microbiol. 2022;60:0244921. https://doi.org/10.1128/jcm.02449-21.

16. Arendrup M, Horn T, Frimodt-Møller N. In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. Infection. 2002;30:286–91.
17. Astvad KMT, Arikan-Akdagli S, Arendrup MC. A pragmatic approach to susceptibility classification of yeasts without EUCAST clinical breakpoints. J Fungi. 2022;8:141.
18. Arendrup MC, Boekhout T, Akova M, Meis JF, Cornely OA, Lortholary O. ESCMID and ECMM joint clinical guidelines for the diagnosis and management of rare invasive yeast infections. Clin Microbiol Infect. 2014;20:76–98.
19. Chen SCA, Perfect J, Colombo AL, Cornely OA, Groll AH, Seidel D, et al. Global guideline for the diagnosis and management of rare yeast infections: an initiative of the ECMM in cooperation with ISHAM and ASM. Lancet Infect Dis. 2021;21:e375–86.
20. Arendrup MC, Jørgensen KM, Guinea J, Lagrou K, Chryssanthou E, Hayette M, et al. Multicentre validation of a EUCAST method for the antifungal susceptibility testing of microconidia-forming dermatophytes. J Antimicrob Chemother. 2020;75:1807–19.
21. Arendrup MC, Meletiadis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, et al. EUCAST technical note on isavuconazole breakpoints for *Aspergillus*, itraconazole breakpoints for *Candida* and updates for the antifungal susceptibility testing method documents. Clin Microbiol Infect. 2016;22:571-e1.
22. Meletiadis J, Leth Mortensen K, Verweij PE, Mouton JW, Arendrup MC. Spectrophotometric reading of EUCAST antifungal susceptibility testing of *Aspergillus fumigatus*. Clin Microbiol Infect. 2017;23:98–103. https://doi.org/10.1016/j.cmi.2016.10.017.
23. Meletiadis J, Efstathiou I, van der Lee HAL, Astvad KMT, Verweij PE, Arendrup MC. Spectrophotometric detection of azole-resistant *Aspergillus fumigatus* with the EUCAST broth microdilution method: is it time for automated MIC reading of EUCAST antifungal susceptibility testing of *Aspergillus* species? J Antimicrob Chemother. 2022;1(5):1296–300.
24. Serrano-Lobo J, Gómez A, Muñoz P, Escribano P, Guinea J. Spectrophotometric azole and amphotericin B MIC readings against *Aspergillus fumigatus* sensu lato using the EUCAST 9.3.2 methodology. Are ≥90 and ≥95% fungal growth inhibition endpoints equally suitable? Med Mycol. 2021;60:1–5. https://doi.org/10.1093/mmy/myab072/6423430.
25. Singh A, Masih A, Khurana A, Singh PK, Gupta M, Hagen F, et al. High terbinafine resistance in trichophyton interdigitale isolates in Delhi, India harbouring mutations in the squalene epoxidase gene. Mycoses. 2018;61:477–84. https://doi.org/10.1111/myc.12772.
26. Ebert A, Monod M, Salamin K, Burmester A, Uhrlaß S, Wiegand C, et al. Alarming India-wide phenomenon of antifungal resistance in dermatophytes: a multicentre study. Mycoses. 2020;63:717–28.
27. Saunte DML, Hare RK, Jørgensen KM, Jørgensen R, Deleuran M, Zachariae CO, et al. Emerging terbinafine resistance in trichophyton: clinical characteristics, squalene epoxidase gene mutations and a reliable EUCAST method for detection. Antimicrob Agents Chemother. 2019;63:1–9.
28. Astvad KMT, Hare RK, Jørgensen KM, Saunte DML, Thomsen PK, Arendrup MC. Increasing terbinafine resistance in danish trichophyton isolates 2019–2020. J Fungi. 2022;8:150.
29. Cañete-Gibas CF, Mele J, Patterson HP, Sanders CJ, Ferrer D, Garcia V, et al. Terbinafine-resistant dermatophytes and the presence of *Trichophyton indotineae* in North America. J Clin Microbiol. 2023;61:0056223.
30. Arendrup MC, Kahlmeter G, Guinea J. How to: perform antifungal susceptibility testing of microconidia-forming dermatophytes following the new reference EUCAST method E.Def 11.0, exemplified by *Trichophyton*. Clin Microbiol Infect. 2021;27:55–60.
31. Guinea J, Verweij PE, Meletiadis J, Mouton JW, Barchiesi F, Arendrup MC, et al. How to: EUCAST recommendations on the screening procedure E.Def 10.1 for the detection of azole resistance in *Aspergillus fumigatus* isolates using four-well azole-containing agar plates. Clin Microbiol Infect. 2019;25:681–7.
32. Meletiadis J, Siopi M, Kanioura L, Jørgensen KM, Perlin DS, Mouton JW, et al. Development and multicentre validation of an agar-based screening method for echinocandin susceptibility testing of *Aspergillus species*. J Antimicrob Chemother. 2019;74:2247–54. https://doi.org/10.1093/jac/dkz154/5491493.
33. Buil JB, van der Lee HAL, Rijs AJMM, Zoll J, Hovestadt JAMF, Melchers WJG, et al. Single-center evaluation of an agar-based screening for azole resistance in *Aspergillus fumigatus* by using VIPcheck. Antimicrob Agents Chemother. 2017;61:10–128.
34. Arendrup MC, Verweij PE, Mouton JW, Lagrou K, Meletiadis J. Multicentre validation of 4-well azole agar plates as a screening method for detection of clinically relevant azole-resistant *Aspergillus fumigatus*. J Antimicrob Chemother. 2017;72:3325–33.
35. Siopi M, Efstathiou I, Arendrup MC, Meletiadis J. Development of an agar-based screening method for terbinafine, itraconazole, and amorolfine susceptibility testing of *Trichophyton* spp. J Clin Microbiol. 2024;62:1–11. https://doi.org/10.1128/jcm.01308-23.
36. Rogers TR, Verweij PE, Castanheira M, Dannaoui E, White PL, Arendrup MC, et al. Molecular mechanisms of acquired antifungal drug resistance in principal fungal pathogens and EUCAST guidance for their laboratory detection and clinical implications. J Antimicrob Chemother. 2022;77:2053–73.
37. Arendrup MC, Patterson TF. Multidrug-resistant *Candida*: epidemiology, molecular mechanisms, and treatment. J Infect Dis. 2017;216:S445-51.
38. Arendrup MC, Perlin DS. Echinocandin resistance: an emerging clinical problem? Curr Opin Infect Dis. 2014;27:484–92.
39. Arendrup MC, Arikan-Akdagli S, Jørgensen KM, Barac A, Steinmann J, Toscano C, et al. European candidaemia is characterised by notable differential epidemiology and susceptibility pattern: results from the ECMM *Candida* III study. J Infect. 2023;87(5):428–37.
40. Arastehfar A, Daneshnia F, Hilmioglu-Polat S, Ilkit M, Yasar M, Polat F, et al. Genetically related

micafungin-resistant *Candida parapsilosis* blood isolates harbouring novel mutation R658G in hotspot 1 of Fks1p: a new challenge? J Antimicrob Chemother. 2021;76:418–22.
41. Thomaz DY, Del Negro GMB, Ribeiro LB, da Silva M, Carvalho GOMH, Camargo CH, et al. A brazilian interhospital candidemia outbreak caused by fluconazole-resistant *Candida parapsilosis* in the COVID-19 era. J Fungi. 2022;8(2):100.
42. Magobo RE, Lockhart SR, Govender NP. Fluconazole-resistant *Candida parapsilosis* strains with a Y132F substitution in the ERG11 gene causing invasive infections in a neonatal unit, South Africa. Mycoses. 2020;63:471–7.
43. Alcoceba E, Gómez A, Lara-Esbrí P, Oliver A, Beltrán AF, Ayestarán I, et al. Fluconazole-resistant *Candida parapsilosis* clonally related genotypes: first report proving the presence of endemic isolates harbouring the Y132F ERG11 gene substitution in Spain. Clin Microbiol Infect. 2022;28:1113–9.
44. Hubler CM, Carvalhaes CG, Castanheira M. Recent increase in fluconazole-nonsusceptible *Candida parapsilosis* in a global surveillance with the expansion of the Erg11 Y132F genotype and a rapid detection method for this alteration. Diagn Microbiol Infect Dis. 2023;106:115957. https://doi.org/10.1016/j.diagmicrobio.2023.115957.
45. Dannaoui E, Espinel-Ingroff A. Antifungal susceptibly testing by concentration gradient strip Etest method for fungal isolates: a review. J Fungi. 2019;5:108.