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A multicentre study to optimize echinocandin susceptibility testing of *Aspergillus* species with the EUCAST methodology and a broth microdilution colorimetric method

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Background: The determination of the minimal effective concentration (MEC) of echinocandins against *Aspergillus* species is subjective, time consuming and has been associated with very major errors.

Methods: The MECs/MICs of 40 WT [10 each of *Aspergillus fumigatus* species complex (SC), *Aspergillus flavus* SC, *Aspergillus terreus* SC and *Aspergillus niger* SC] and 4 non-WT *A. fumigatus* isolates were determined with EUCAST E.Def 9.3.1 read microscopically, macroscopically, spectrophotometrically and colorimetrically in three centres. The optimal conditions for spectrophotometric (single- versus multi-point readings) and colorimetric (XTT/menadione concentration and stability, incubation time) methods were evaluated in preliminary studies using different cut-offs for the determination of macroscopic, spectrophotometric and colorimetric MIC endpoints compared with the microscopically determined MEC. Inter-centre and inter-method essential (within one 2-fold dilution) agreement (EA) and categorical agreement (CA) were determined.

Results: Both macroscopic and spectrophotometric endpoint readings showed poor inter-centre EA (53%–66%) and low CA (41%–88%) in distinguishing WT from non-WT *A. fumigatus* SC isolates, while significant differences compared with the microscopic MECs were observed for all echinocandins (EA 6%–54%). For the colorimetric method, the optimal conditions were 400 mg/L XTT/6.25 μM menadione, incubation for 1–2 h until the drug-free control reached an absorbance at 450/630 nm of >0.8 and use of 50% inhibition of XTT conversion as a cut-off for all species and echinocandins. All non-WT isolates had high XTT MICs >1 mg/L, whereas the overall inter-centre EA and CA were 72%–89% and 100%, respectively.

Conclusions: The XTT colorimetric assay improved the antifungal susceptibility testing of echinocandins against *Aspergillus* spp., reliably detecting non-WT isolates.

Introduction

Echinocandins target the fungal cell wall by blocking the synthesis of β-(1,3)-D-glucan. They are fungicidal against *Candida*, but fungistatic and less efficacious compared with amphotericin B and voriconazole against *Aspergillus* infections. However, caspofungin or micafungin are indicated in therapy-refractory or -intolerant patients with invasive aspergillosis and in combination with other systemic antifungal agents in settings where azole resistance is prevalent or suspected.[1,2] With increasing azole resistance in *Aspergillus fumigatus* reported globally, the growing use of echinocandins may result in emerging echinocandin resistance. Indeed, *Aspergillus* spp. isolates with reduced susceptibility to echinocandins as well as breakthrough infections emerging during echinocandin treatment of aspergillosis have been reported,[3–11] highlighting the need for reliable *in vitro* susceptibility testing in the routine clinical laboratory.

Broth microdilution (BMD) methods, standardized by CLSI and EUCAST, are considered the gold standard for evaluating the *in vitro* antifungal susceptibility of moulds.[12,13] The recommended endpoint for determining echinocandin activity against

filamentous fungi is the minimal effective concentration (MEC), defined as the lowest drug concentration with aberrant growth as a result of altered multibranched rosette morphology of mycelia. However, this reading is not only labour intensive but also subjective, which even for trained staff members may be difficult to perform in a reproducible manner.[7] Hence, the current reference methodology is not easily incorporated into daily laboratory practice and reduced susceptibility to echinocandins may be underdiagnosed. Furthermore, great concern has arisen since variation has been observed in published distributions of echinocandin MECs of *Aspergillus* spp.[14–16] that may impede the accurate identification of non-WT isolates.[7]

At the same time, colorimetric readings using multiple reading points per well and employing the dye MTT have been used to quantify fungal growth after exposure to caspofungin and an MIC endpoint close to the CLSI MEC was determined.[17] Moreover, colorimetric MIC endpoints were further optimized for amphotericin B, azole[18] and caspofungin[19] susceptibility testing of different *Aspergillus* spp. using a colorimetric assay based on the water-soluble tetrazolium salt XTT. XTT is used to assess cell viability as actively respiring cells convert the water-soluble XTT to a water-soluble, orange-coloured formazan product, which facilitates automated MIC reading and provides reproducible quantitative results. Similar methods for all three echinocandins in accordance with the EUCAST recommendations have not yet been described, whereas multicentre evaluation of the above-mentioned CLSI-based methods has not been performed.

The aim of this multicentre study was to develop and validate a reproducible EUCAST method for echinocandin susceptibility testing of *Aspergillus* spp. that reliably differentiates WT and non-WT isolates. For this purpose, a spectrophotometric method assessing fungal biomass and a colorimetric method assessing metabolic activity (MA) and viability were evaluated.

Materials and methods

Isolates

A total of 40 WT clinical isolates of *Aspergillus* spp. [10 each of *A. fumigatus* species complex (SC), *Aspergillus flavus* SC, *Aspergillus terreus* SC and *Aspergillus niger* SC] retrieved from the strain collection of Centre 1 and 4 non-WT *A. fumigatus* isolates from D. Perlin's laboratory (DPL) collection possessing elevated MEC values, with (DPL1035-homo[20] and DPL24053[3]) or without (DPL55985 and DPL32458) known *fks* alterations, were tested. Species identification of WT strains was based on the morphological characteristics of the colonies and microscopy. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains. The isolates were stored in normal sterile saline with 10% glycerol at −70°C until use.

Antifungal drugs, chemical reagents and medium

Laboratory-grade standard powders of anidulafungin (Pfizer, Inc., Groton, CT, USA), caspofungin acetate (Merck & Co., Inc., Whitehouse, NJ, USA) and micafungin (Astellas Pharma, Inc., Tokyo, Japan) were dissolved in DMSO (Chem-Lab NV, Zedelgem, Belgium) and stock solutions of 10 mg/mL were stored in small portions at −70°C. XTT sodium salt (AppliChem, Darmstadt, Germany) was dissolved in sterile water before use. Menadione (MEN; Sigma–Aldrich, Steinheim, Germany) was dissolved in absolute ethanol (VWR Chemicals, Fontenay-sous-Bois, France) and stock solutions of 58×10^{-3} M were stored at −70°C. The medium used throughout was RPMI 1640 medium (with L-glutamine, without bicarbonate) (AppliChem, Darmstadt, Germany) buffered to pH 7.0 with 0.165 M MOPS (AppliChem, Darmstadt, Germany) and supplemented with 2% glucose (AppliChem, Darmstadt, Germany).

BMD susceptibility testing

The reference BMD procedure was performed according to the EUCAST guidelines.[13] Briefly, each isolate was revived by subculturing it twice on Sabouraud dextrose agar plates with gentamicin and chloramphenicol (SGC2; bioMérieux) at 30°C for 5–7 days and conidial suspensions were prepared in sterile water with 0.1% Tween 20. Two-fold serial drug concentrations ranging from 0.001 to 1 mg/L anidulafungin and micafungin and 0.004 to 4 mg/L caspofungin (for the non-WT *A. fumigatus* isolates, 0.5 to 64 mg/L for all three echinocandins) were used. The plates were incubated at 37°C for up to 48 h.

Study design

Initial optimization of test conditions was conducted in Centre 1 using 40 echinocandin WT and 4 molecularly characterized non-WT *Aspergillus* spp. strains. Subsequently, pre-weighed pure antifungals and chemical reagents as well as all previously tested *Aspergillus* spp. isolates were sent blinded to Centre 2 and Centre 3, accompanied by step-by-step instructions for the evaluation of the methods' performance in a three-laboratory multicentre setting (Greece, Denmark and the Netherlands). All *Aspergillus* spp. isolates were tested in three centres by experienced mycologists with all four methods (macroscopic, microscopic, spectrophotometric and colorimetric) following the optimized conditions. In each participating laboratory all plates were read by an experienced reader, blinded to the susceptibility phenotype of the strains.

Microscopic method

The MEC was defined as the lowest echinocandin concentration at which short, stubby and highly branched hyphal clusters were observed, using an inverted microscope, compared with the growth control well after 24 and 48 h of incubation.

Macroscopic method

Fungal growth (FG) in each well was determined by macroscopic inspection of the plate after 24 and 48 h of incubation, with the aid of a magnifying mirror, and the MIC was defined with five endpoints as the highest drug concentration with no growth (score 0), slight growth (~75% growth inhibition, score 1), prominent reduction of growth (~50% growth inhibition, score 2), slight reduction of growth (~25% growth inhibition, score 3) and no inhibition of growth (score 4) compared with growth of the drug-free control.

Spectrophotometric method

The OD of each well was measured spectrophotometrically at 405 nm as at this wavelength hyphae have the highest OD[21] at either a single point (centre of the well) or 10 points across the horizontal dimension after 24 and 48 h of incubation and the percentage of FG (%FG) was calculated for each well as $\%FG = (OD_{drug\ well} - OD_{background\ drug\ well})/(OD_{drug\text{-}free\ well} - OD_{background\ drug\text{-}free\ well}) \times 100$. The spectrophotometric MICs were determined as the lowest drug concentrations corresponding to different percentage of growth inhibition (100%−%FG) compared with the drug-free control.

Colorimetric method

Preliminary studies were carried out in order to determine the optimal concentrations of XTT/MEN and incubation period that produced a well-defined

concentration–effect curve with a decline in MA observed at the MEC. In particular, different concentrations of XTT (100, 200, 400 and 600 mg/L) and MEN (1.25, 6.25, 12.5 and 25 μM) were used in plates incubated for 24 and 48 h. The absorbance (ABS) at 450/630 nm was measured after 1–5 h of incubation with 50 μL of XTT/MEN solution (5×) in each well. The %MA assessed by %XTT conversion was calculated for each well as $\%MA = (ABS_{drug\ well} - ABS_{background\ drug\ well})/(ABS_{drug\text{-}free\ well} - ABS_{background\ drug\text{-}free\ well}) \times 100$. The colorimetric MICs were determined as the lowest drug concentrations corresponding to different percentage of inhibition of MA (100%−%MA) compared with the drug-free control.

Inter-experimental variation was evaluated by testing selected isolates of each species (27–32 isolates, 5–10 isolates/species). Experiments were performed independently on two different days under the same conditions with individually prepared inocula. In addition, stability of XTT solution stored at 4°C and −70°C either alone or with MEN was assessed with selected isolates of each species up to 6 months. Parameters that could potentially affect the performance of the XTT-based assay [once- or twice-subcultured isolates, with or without filtration of the inoculum, adjustment of the suspension by counting the conidia in a haemocytometer chamber or by using a spectrophotometer, RPMI 1640 medium provided by different manufacturers (AppliChem versus Sigma–Aldrich) and incubation temperature of 35°C versus 37°C] were evaluated with selected isolates of each species.

Analysis

MIC endpoints were compared with MECs between WT and non-WT *A. fumigatus* isolates in order to find the best MIC endpoint and method that separated the two groups. The inter-method essential agreement (EA) between the reference microscopic MEC method and the macroscopic, spectrophotometric and colorimetric MIC methods was calculated for each echinocandin and species as the percentage of isolates with MICs within one 2-fold dilution of the microscopic MEC for all three participating centres. The inter-centre EA of each method was calculated for each echinocandin and species as the percentage of isolates with microscopic MEC and macroscopic, spectrophotometric and colorimetric MICs within one 2-fold dilution of the median (among the three centres) MEC and MICs, respectively. The differences between the inter-centre EA of the reference microscopic MEC method and the macroscopic, spectrophotometric and colorimetric MIC methods was assessed with paired *t*-test for all echinocandins ($P < 0.05$ was considered statistically significant). Categorical agreement (CA) in distinguishing non-WT from WT *A. fumigatus* SC isolates was calculated for all centres and each echinocandin. The inter-centre variation of the method with the highest inter-centre EA and CA was estimated by calculating the sum of the absolute $\log_2$ differences between the MIC of each centre and the median MIC and compared with the inter-centre variation of the microscopic MEC determination.

Results

Microscopic method

The determination of MECs with the inverted microscope was quite challenging since the differences by microscopy were subtle (Figure 1). In particular, no clear echinocandin-induced morphological hyphal alterations could be defined with the exception of *A. flavus* SC isolates. For the other species, MEC values were reported as the lowest concentration where differences in the hyphal density were observed. For the WT isolates, the median (range) MECs were 0.016 (0.001–0.125), 0.5 (0.125–1) and 0.03 (0.002–0.125) mg/L (Centre 1), 0.03 (0.016–0.125), 0.25 (0.06–0.5) and 0.03 (0.008–0.125) mg/L (Centre 2) and 0.008 (0.004–0.016), 0.125 (0.125–0.25) and 0.016 (0.004–0.03) mg/L (Centre 3) for anidulafungin, caspofungin and micafungin, respectively. The distributions of MECs for anidulafungin (0.001–0.125 mg/L) and micafungin (0.002–0.125 mg/L) spanned seven–eight 2-fold dilutions and were wider than those of caspofungin (0.06–1 mg/L, five 2-fold dilutions) mainly because among the four species, *A. terreus* SC had the lowest MECs for anidulafungin (0.001–0.06 mg/L) and micafungin (0.002–0.06 mg/L). All non-WT isolates had high MECs (>1 mg/L) for all echinocandins. The overall EA between the centres was 72% for anidulafungin, 88% for caspofungin and 83% for micafungin (Table 1). Of note, although the most significant echinocandin-induced morphological changes were found for *A. flavus* SC, the highest MEC discrepancies were also observed for this species, emphasizing the inter-laboratory variability and unsatisfactory reproducibility of the method. The CA in distinguishing WT from non-WT *A. fumigatus* SC isolates was 100% for all three echinocandins and centres (Table 1). The MIC values of anidulafungin and micafungin for the quality control isolates were within the reference ranges.

Macroscopic method

Macroscopic evaluation of growth in each well mostly resulted in growth scores ranging from 3 at high concentrations to 4 at low concentrations of all echinocandins (Figure 2). Growth scores down to 1 were also found for some isolates, particularly of *A. niger*. Notably, for some WT isolates no decrease in turbidity was observed at any concentration, whereas slight growth inhibition (score 3) was found for some non-WT isolates at low concentrations, indicating that macroscopic assessment is not reliable for echinocandin susceptibility testing of *Aspergillus* spp. The highest agreement for macroscopic MICs was found with score 3 (slight growth inhibition) for all echinocandins. In particular, the levels of agreement between the microscopic and macroscopic (score 3) methods for all centres was 26% for anidulafungin (54% in Centre 1, 13% in Centre 2 and 13% in Centre 3), 54% for caspofungin (73% in Centre 1, 30% in Centre 2 and 58% in Centre 3) and 29% for micafungin (54% in Centre 1, 10% in Centre 2 and 23% in Centre 3) (Table 1). The overall inter-centre EA was 53% for anidulafungin, 58% for caspofungin and 58% for micafungin (Table 1) and it was significantly lower than the microscopic MEC determination for all echinocandins (*t*-test $P = 0.0002$). The average CA in distinguishing WT from non-WT *A. fumigatus* SC isolates was 78%, 82% and 88% for anidulafungin, caspofungin and micafungin (range 63%–100% among centres), respectively, as several WT strains were misclassified as non-WT (Table 1).

Spectrophotometric method

The median (range) OD increase of drug-free control after 24/48 h of incubation was low: 0.17 (0.08–0.53)/0.30 (0.15–0.42) for Centre 1, 0.09 (0.03–0.19)/0.22 (0.12–0.37) for Centre 2 and 0.04 (0–0.11)/0.13 (0.04–0.26) for Centre 3. The single- and multi-point spectrophotometric readings of the wells did not produce a clear-cut endpoint since the percentage of growth inhibition compared with the drug-free control ranged from 0% to 50% for most WT and non-WT isolates, making distinguishing between them impossible (Figure 3). Using different cut-offs of growth inhibition (10%–50%), the levels of agreement between the microscopic and spectrophotometric methods and between the centres never

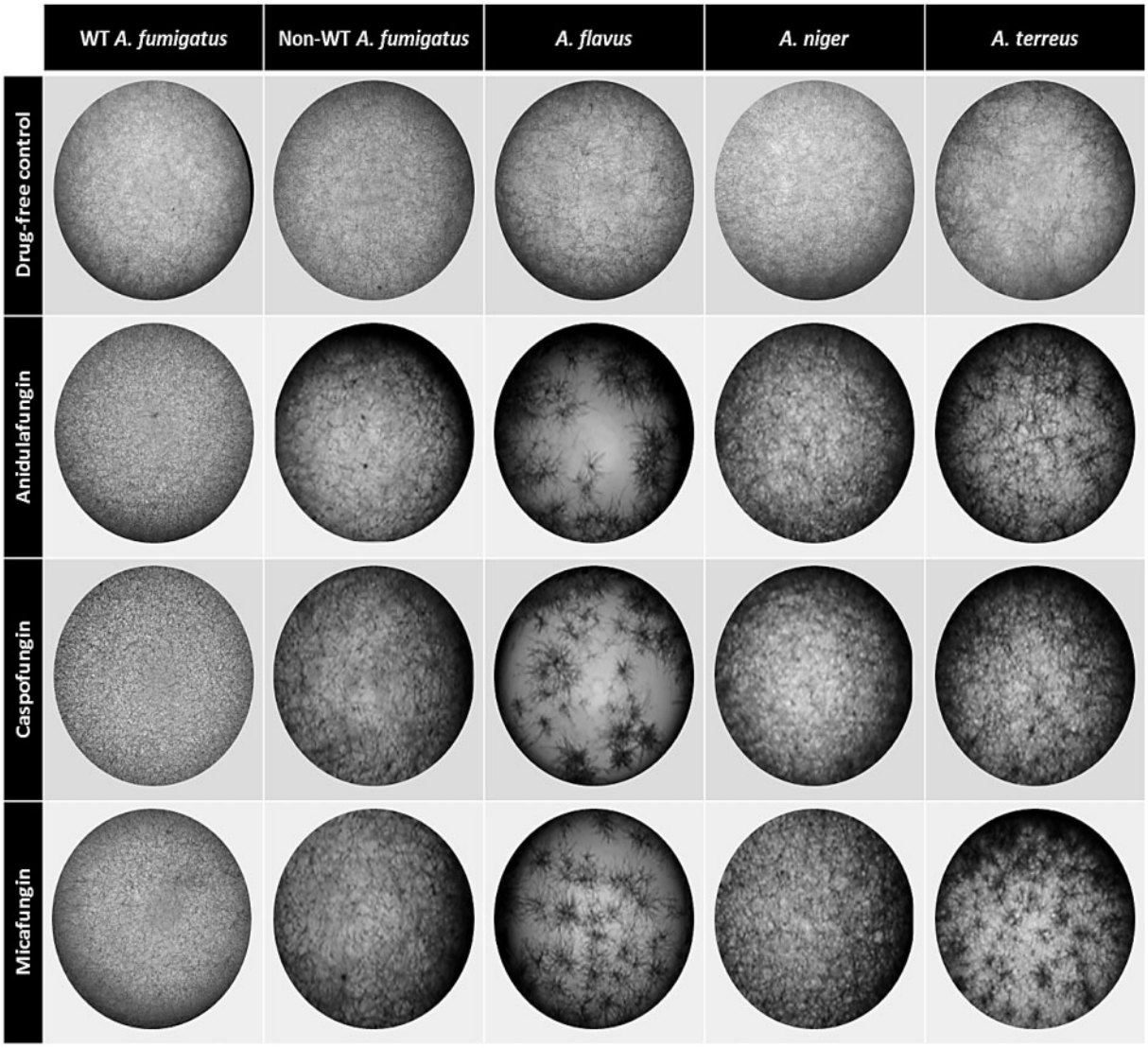

Figure 1. Microscopy images at concentrations of echinocandins adjacent to the MEC (one dilution higher) against the WT *Aspergillus* spp. isolates (8 mg/L for the non-WT isolate).

exceeded 20% and in most cases ranged between 5% and 10%, indicating that the spectrophotometric assessment of growth does not reflect the microscopic changes caused by echinocandins. In particular, the highest agreement for spectrophotometric MICs was found using the 50% cut-off growth inhibition. The levels of agreement between the microscopic and spectrophotometric (50% growth inhibition) methods for all centres were: 7% for anidulafungin (12% in Centre 1, 3% in Centre 2 and 5% in Centre 3), 12% for caspofungin (10% in Centre 1, 13% in Centre 2 and 15% in Centre 3) and 6% for micafungin (12% in Centre 1, 3% in Centre 2 and 3% in Centre 3) (Table 1). The overall EA between the centres was 61% for anidulafungin, 66% for caspofungin and 66% for micafungin (Table 1) and was significantly lower than for the microscopic MEC determination for all echinocandins (*t*-test $P = 0.0067$). The average CA in distinguishing WT from non-WT *A. fumigatus* SC isolates was 44%, 41% and 42% for anidulafungin, caspofungin and micafungin (range 14%–85% among centres), respectively, since a number of WT strains were wrongly classified as non-WT and vice versa (Table 1).

Colorimetric method

The combination of 400 mg/L XTT and 6.25 μM MEN was found to be optimal in distinguishing WT from non-WT isolates in pilot studies (data not shown) and was further evaluated in all three centres with incubation of 1, 2 and 3 h with XTT/MEN solution. Colour production in the drug-free control reached a median (range) $ABS_{450/630}$ after 1 h and 2 h of 1.61 (0.64–2.80) and >3.00 (1.70 to >3.00) in Centre 1, 0.41 (0.08–1.02) and 1.13 (0.23–2.27) in Centre 2 and 0.55 (0.16–1.24) and 1.51 (0.63–3.6) in Centre 3. Further incubation to 3 h did not increase XTT conversion. Notably, significant inter-strain and inter-centre variation in colour production was found. However, all non-WT isolates converted XTT, even at high concentrations of echinocandins, resulting in <50% colour

Table 1. Overall levels of inter-centre/inter-method EA (within one 2-fold dilution) and CA of the reference (microscopically defined MECs), macroscopic (based on score 3; ∼25% growth inhibition), spectrophotometric (based on 50% growth inhibition) and colorimetric (based on 50% inhibition of XTT conversion) methods for *Aspergillus* spp.

			Inter-centre/inter-method[a] EA (CA), %			
Species	No. of isolates	Antifungal	microscopic	macroscopic	spectrophotometric	colorimetric
A. fumigatus SC	14	AFG	83/REF (100)	50/9 (78)	57/0 (44)	90/25 (100)
		CAS	87/REF (100)	57/48 (82)	77/7 (41)	90/72 (100)
		MFG	100/REF (100)	60/12 (88)	60/3 (42)	100/29 (100)
A. flavus SC	10	AFG	60/REF	53/33	67/3	73[b]/34
		CAS	87/REF	47/50	70/17	93/68
		MFG	77/REF	60/53	80/3	80/17
A. terreus SC	10	AFG	53/REF	53/30	60/17	70[b]/53
		CAS	90/REF	50/37	60/13	90/52
		MFG	63/REF	60/17	63/7	83/58
A. niger SC	10	AFG	90/REF	57/33	60/7	77[b]/60
		CAS	87/REF	77/80	57/13	97/83
		MFG	90/REF	53/33	60/10	73[c]/44
Total	44	AFG	72/REF	53/26	61/7	72/43
		CAS	88/REF	58/54	66/12	89/69
		MFG	83/REF	58/29	66/6	78/37

AFG, anidulafungin; CAS, caspofungin; MFG, micafungin.
[a]The percentage EA between MICs of each method compared with the reference (REF) microscopic MECs was determined.
[b]Higher inter-centre EA (87% for *A. flavus* and *A. terreus* and 83% for *A. niger*) was found with 40% inhibition of XTT conversion.
[c]Higher inter-centre EA (87%) was found with 60% inhibition of XTT conversion.

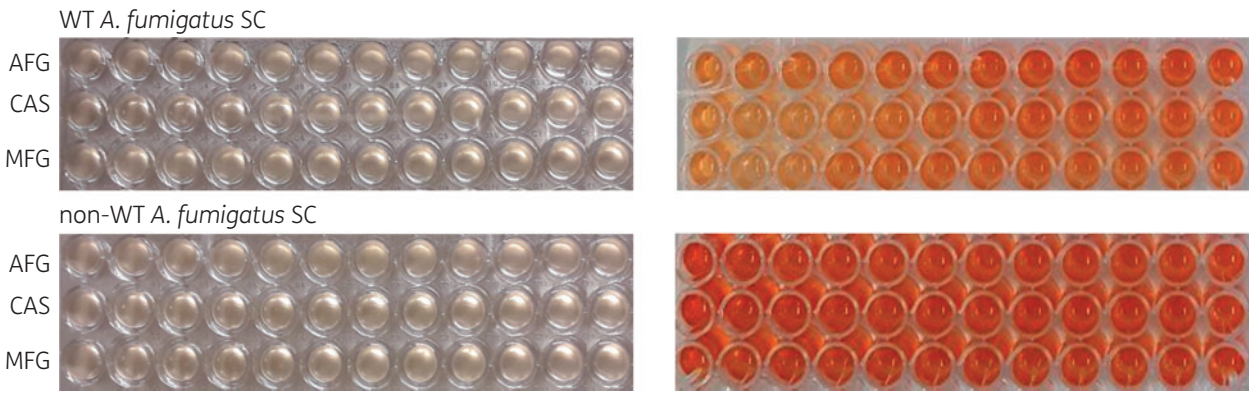

Figure 2. Macroscopic evaluation of growth (left) and XTT conversion (right) by a WT *A. fumigatus* SC (top) and a non-WT *A. fumigatus* (bottom) isolate, respectively, in the presence of different concentrations of anidulafungin (AFG) and micafungin (MFG) ranging from (left to right) 1 to 0.001 mg/L and caspofungin (CAS) ranging from 4 to 0.004 mg/L. The last column represents the drug-free growth control. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

inhibition at the highest echinocandin concentration in all three centres (Figures 2 and 3). In contrast, prominent inhibition of colour production was observed at the lower concentrations of all echinocandins for the WT isolates (Figures 2 and 3). The colour inhibition endpoints for optimal EA between the microscopic MECs and the colorimetric MICs differed considerably among species, drugs and centres. Overall for all centres, the best EA between microscopic MECs and colorimetric MICs was found with the 50% colour inhibition endpoint (43% for anidulafungin, 69% for caspofungin and 37% for micafungin) although variable levels of EA among the species were found (25%–60% for anidulafungin, 52%–83% for caspofungin and 17%–58% for micafungin) (Table 1).

Since microscopic MEC determination may be subjective and questionable as a gold standard, the overall agreement among XTT MICs of the three centres using different cut-off levels of inhibition of MA for each species and echinocandin was calculated. The 50% inhibition of XTT conversion yielded the highest EA among the three centres for all species providing that an $ABS_{drug\text{-}free\ control}$ of >0.8 was obtained, i.e. 1 h for Centre 1 and 2 h for Centre 2 and Centre 3 (Figure 3). For anidulafungin, although the 50% inhibition of XTT conversion cut-off resulted in 10% lower inter-centre

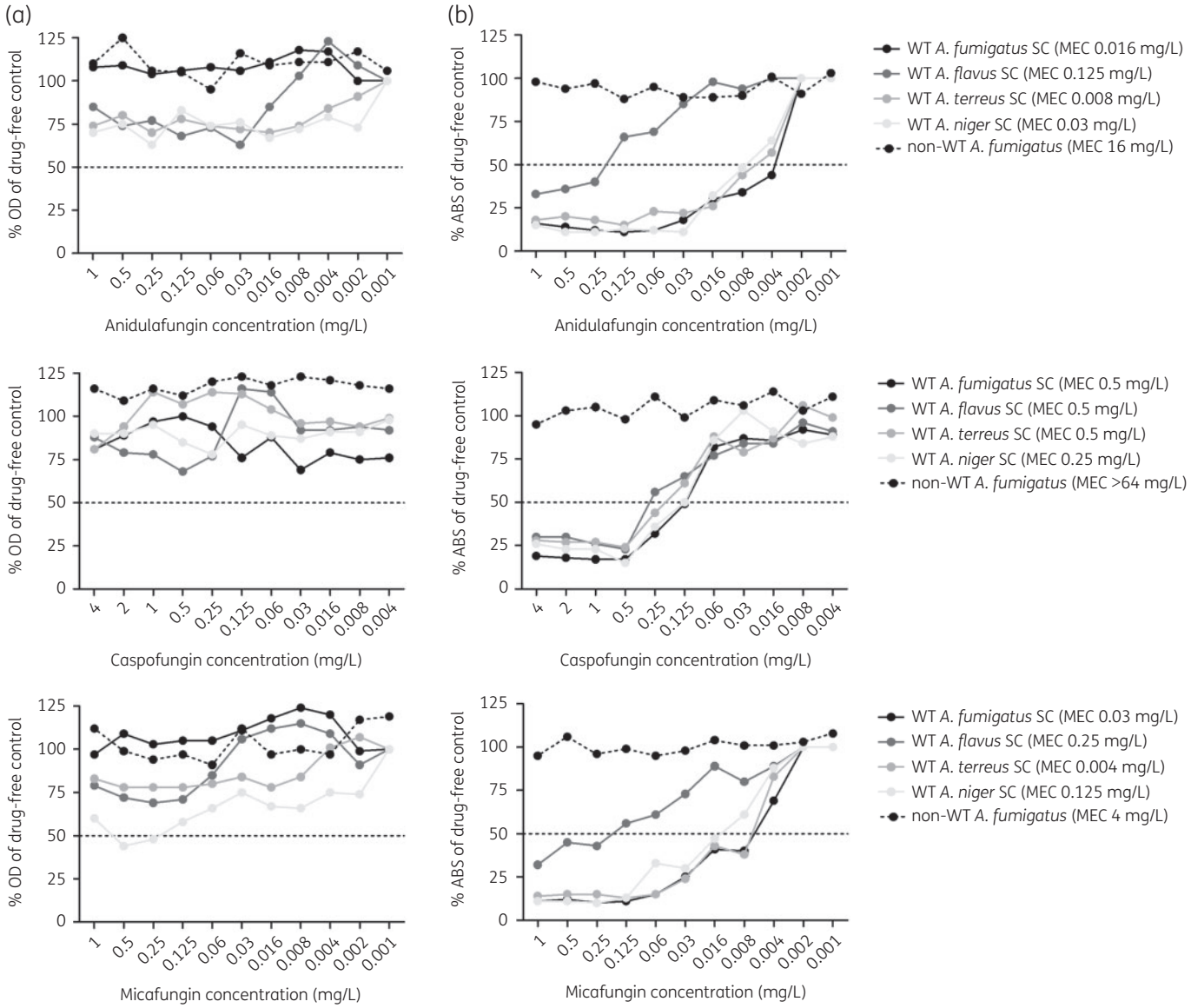

Figure 3. Spectrophotometric (a) and colorimetric (b) concentration–effect curves for the three echinocandins based on the OD at 405 nm and the ABS after XTT conversion by viable fungi measured at 450/630 nm (400 mg/L XTT/6.25 μM MEN, incubation 1–2 h until drug-free control reached an ABS >0.8), respectively.

agreement than the 40% inhibition of XTT conversion cut-off (72% versus 82%, respectively) overall for all species, for *A. fumigatus* the 50% cut-off yielded higher levels of agreement than the 40% cut-off (90% versus 87%, respectively). Since most clinical isolates are *A. fumigatus* and since acquired echinocandin resistance has not yet been reported in non-*A. fumigatus* species to our knowledge, we propose the 50% XTT conversion inhibition cut-off for all echinocandins.

The median XTT MIC_{50} endpoints of anidulafungin (0.002–0.004 mg/L), caspofungin (0.125–0.5 mg/L) and micafungin (0.004–0.008 mg/L) for the WT *A. fumigatus* isolates and of caspofungin for all the other species (0.125–0.5 mg/L) were similar across the centres. For the non-*A. fumigatus* species, the anidulafungin and micafungin median XTT MIC_{50}s determined at Centre 2 (0.016 and 0.03 mg/L, respectively) were two–three 2-fold dilutions higher than in Centre 1 and Centre 3 (0.002 and 0.004–0.008 mg/L, respectively). All non-WT isolates had high colorimetric MICs (>1 mg/L) in all three centres. Excellent EA (100%) was found for the non-WT *A. fumigatus* isolates, while the overall inter-centre EA for all isolates was 72% for anidulafungin, 89% for caspofungin and 78% for micafungin (Table 1). No statistically significant differences with the microscopic MEC determination were found for any of the echinocandins (*t*-test $P = 0.22$). The CA in distinguishing WT from non-WT *A. fumigatus* SC isolates was 100% for all three echinocandins and centres (Table 1). The inter-centre variability of the colorimetric method was 42%–47% lower than the inter-centre variability of the microscopic method (58, 43 and 53 versus 109, 74 and 92 sum of absolute

log_2 differences for anidulafungin, caspofungin and micafungin for the two methods, respectively).

Two centres (1 and 3) repeated XTT experiments for 27–32 (5–10 isolates/species) isolates and reproducibility was 70%–90%. The EA between 1 h and 2 h XTT endpoints was 87% for caspofungin and 78%–93% for anidulafungin and micafungin.

When the XTT solution was stored at 4°C, either alone or with MEN, a 50% loss of efficacy was observed at Day 15, whereas it was stable for up to 6 months at −70°C. The performance of the colorimetric method was not influenced (zero−one 2-fold dilution differences) by any of the parameters tested (once- or twice-subcultured isolates, filtration of the inoculum, adjustment of the suspension by haemocytometer chamber or spectrophotometer, RPMI 1640 medium provider or incubation temperature of 35°C or 37°C).

Discussion

Although correct discrimination between echinocandin WT and non-WT *A. fumigatus* was obtained using the gold standard BMD EUCAST E.Def. 9.3.1 method with microscopic endpoint reading as recommended, this method is difficult to read and implement in the routine laboratory. Consequently, echinocandin susceptibility testing of *Aspergillus* isolates either is not performed or is only conducted in reference centres. The present multicentre study confirmed that the standard microscopic MEC determination of EUCAST E.Def. 9.3.1 is quite challenging, yielding considerable inter-centre variation even for experienced mycologists. We moreover showed that both macroscopic and spectrophotometric endpoint readings of EUCAST E.Def. 9.3.1 plates showed poor overall inter-centre EA (53%–66%) with significant differences compared with the microscopic MEC determination for all echinocandins, as well as low CA (41%–88%) in distinguishing WT from non-WT isolates, and thus are not valid alternative methods for MEC determination. Taking into account the potential for increased objectivity, a colorimetric XTT assay for echinocandin susceptibility testing of *Aspergillus* spp. was developed and validated. The optimal test conditions were 400 mg/L XTT/6.25 μM MEN and incubation until an ABS at 450/630 nm of >0.8 of drug-free control was reached. The colorimetric method could easily detect non-WT isolates since these resulted in <50% inhibition of XTT conversion compared with the drug-free control at high concentrations of all three echinocandins. High inter-centre CA (100%) and EA (>70% for all species, >90% for *A. fumigatus* SC) was found among XTT MICs thus reducing inter-centre MIC variation by 42%–47%.

CLSI and EUCAST echinocandin susceptibility testing methods for *Aspergillus* spp. are based on broth dilution and are technically demanding, which is reflected in the variability of published MEC ranges.[14–16] Notably, they may not reliably identify non-WT isolates, as was demonstrated in a study for a multi-azole-resistant *A. fumigatus* isolate from a patient failing itraconazole and caspofungin therapy.[7] That isolate was found to be echinocandin WT by CLSI and EUCAST BMD methods, but non-WT by Etest and *in vivo* in a haematogenous mouse model. Reduced susceptibility was linked to *fks* gene up-regulation and no *fks* mutations were found. Obviously, this raises great concern if the current reference methodology clearly and reliably separates isolates with resistance mechanisms from isolates without. With the increasing number of reports on azole resistance in *Aspergillus* spp., the echinocandin class of drug becomes increasingly important and more often used (as salvage or combination therapy)—a scenario where reliable susceptibility testing is of utmost importance.

For the EUCAST methodology specifically, where a high inoculum of 10^5 cfu/mL is used (as compared with the CLSI method) the determination of MEC of echinocandins is particularly challenging because of heavy growth after 48 h. It is difficult to identify short aberrant mycelia in drug-containing wells when growth is more or less confluent. Subtle differences of growth between wells with different concentrations of echinocandins cannot be detected macroscopically or microscopically even when multiple reading points per well were used. Despite these subtle differences in growth, significant differences (>50%) in metabolism in drug-free and drug-containing wells were detected with the XTT method after 24 h of incubation. No XTT conversion was observed at echinocandin concentrations within the MIC ranges of WT isolates, facilitating the detection of non-WT strains with an objective, fast and automated method. The XTT method could also differentiate WT isolates with different MECs in a reproducible manner among different laboratories provided that drug-free controls attained sufficient MA (ABS > 0.8) within 1–2 h of incubation with XTT. The XTT/MEN 'ready to use' solution could be stored at −70°C to increase stability and used after MEC was determined with the standard approach in the EUCAST plate. Furthermore, inter-laboratory agreement may be increased further when a standardized inoculum is used either via filtration (as used in Centre 3) or via microscopic observation and counting in a haemocytometer (as used in Centre 1).

This study has strengths and limitations. The strengths are that the study was performed in laboratories with mycology expertise and that the same plates were used for macroscopic, spectrophotometric and colorimetric endpoints, thus avoiding differences in inoculum or viability of the inoculum to affect intra-centre comparisons. The major limitation is that non-WT isolates with more modest resistance were not available and hence the CA may be overestimated (possibly particularly so for the methods with subjective endpoint reading).

In summary, to be of any value, an *in vitro* susceptibility test must be reproducible and able to detect resistance. The echinocandin XTT assay for *Aspergillus* spp. developed in the present study is quite simple and it avoids the need for special training or expertise associated with the macroscopic/microscopic endpoint reading for the reference BMD method. Indeed, calculation of the %MA can be performed automatically through a predefined protocol in a plate-reading spectrophotometer, allowing an objective determination of the XTT MIC using the proposed cut-off values of inhibition of XTT conversion.

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