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In vitro activity of isavuconazole against 208 *Aspergillus flavus* isolates in comparison with 7 other antifungal agents: assessment according to the methodology of the European Committee on Antimicrobial Susceptibility Testing☆,☆☆

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Abstract

Aspergillus flavus is the second most common species causing invasive aspergillosis after *A. fumigatus*. In certain countries like India, Sudan, and Saudi Arabia, *A. flavus* is most frequently isolated from patients with fungal rhinosinusitis and endophthalmitis. *A. flavus* exhibit an increased resistance to antifungal agents compared to *A. fumigatus.* We determined the in vitro activity of isavuconazole, voriconazole, posaconazole, itraconazole, amphotericin B, caspofungin, micafungin, and anidulafungin against 208 isolates of *A. flavus* by the EUCAST method and compared with the results obtained by the CLSI method. Isavuconazole and voriconazole MICs were ≤2 μg/mL in 99% and 95%, respectively. Posaconazole and itraconazole MICs were ≤0.5 and ≤1 μg/mL, respectively, for all isolates. MICs of amphotericin B were ≥2 μg/mL in 91%; 36% of them exhibited MICs of ≥8 μg/mL. All echinocandins demonstrated good anti–*A. flavus* activity. The essential agreement of the MIC/MEC results by EUCAST with CLSI broth dilution method assessed at ±2 dilutions was good for itraconazole (97.8%), voriconazole (100%), posaconazole (98.3%), isavuconazole (98.9%), caspofungin (99.4%), and anidulafungin (100%), but poor for amphotericin B (53.5%) and micafungin (79.1%).
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1. Introduction

Invasive aspergillosis poses a serious threat to immunocompromised patients worldwide, causing high morbidity and mortality (Denning, 1998). Among the more than 300 species of *Aspergillus* known, only a few species are known to cause infection in humans (Balajee et al., 2009; Denning, 1998). *A. fumigatus* is considered to be the commonest causative agent of invasive aspergillosis (Balajee et al., 2009; Denning, 1998; Morgan et al., 2005). However, in certain countries including India, Sudan, and Saudi Arabia, *A. flavus* is most frequently isolated from patients with fungal rhinosinusitis and endophthalmitis (Hedayati et al., 2007; Pasqualotto, 2009). *A. flavus* has also been reported in a few studies to be more virulent and exhibits an increased resistance to antifungal agents compared to *A. fumigatus* (Ford and Friedman, 1967; Hedayati et al., 2007).

Several antifungal agents including lipid formulations of amphotericin B, caspofungin, micafungin, voriconazole, itraconazole, and posaconazole have been used for the treatment of invasive aspergillosis. However, voriconazole is recommended as the drug for primary therapy (Herbrecht

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et al., 2002; Walsh et al., 2008). In 2006, resistance to azoles was reported in 2% of *A. fumigatus* strains during a 3-month survey (Klaassen et al., 2010). A few centers reported even higher rates of azole resistance of 10–15% (Verweij et al., 2009). The development of acquired resistance during azole therapy is still not clear. However, clinical data suggesting the development of azole resistance during and after azole therapy have been reported (Howard et al., 2009). Furthermore, clinical failures in patients with invasive aspergillosis occur commonly (Howard et al., 2009; Verweij et al., 2007).

Isavuconazole (ISA; BAL4815) is a new broad-spectrum triazole agent in late stage of clinical development for the treatment of invasive candidosis and aspergillosis (Guinea et al., 2008; Warn et al., 2006). The in vitro activity of ISA has been tested against a large collection of *A. fumigatus* isolates (Guinea et al., 2008; Warn et al., 2006; Yamazaki et al., 2010). However, only a few studies have reported the activity of ISA against *A. flavus* (De La Escalera et al., 2008; Guinea et al., 2008; Warn et al., 2006; Yamazaki et al., 2010). Here, we report the in vitro activities of ISA and 7 comparators against 208 isolates of *A. flavus* from India and The Netherlands according to the methodology of the European Committee on Antimicrobial Susceptibility (EUCAST). In addition, the results obtained with EUCAST were compared with the results obtained using the same strains by the CLSI broth microdilution method (Shivaprakash et al., 2011).

2. Materials and methods

2.1. Fungal isolates

A total of 208 clinical and environmental *A. flavus* isolates were used in the study. The isolates of Indian origin were obtained from the National Culture Collection of Pathogenic Fungi (NCCPF), Postgraduate Institute of Medical Education and Research, Chandigarh, India (n = 180). The Dutch isolates were from the Canisius Wilhelmina Hospital, Nijmegen, The Netherlands (n = 24), and the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands (n = 4). The clinical origin of the isolates (n = 196) is detailed in Table 1. The environmental isolates (n = 12) were obtained either from the CBS collection (n = 4, CBS 116416; CBS 121703; CBS100927 [neotype of *A. flavus*]; CBS 573.65) or from outdoor sources from India (n = 2) and The Netherlands (n = 6). The identity of all the isolates was confirmed by using conventional procedures (De Hoog et al., 2009), DNA sequencing of the ITS region of rDNA, and amplified fragment length polymorphism analysis (De Valk et al., 2007; Rudramurthy et al., 2011).

2.2. Antifungal agents

Amphotericin B (AMB; Bristol Myers Squibb, Woerden, The Netherlands), voriconazole (VOR; Pfizer Central Research, Sandwich, Kent, United Kingdom), itraconazole (ITR; Janssen Cilag, Tilburg, The Netherlands), posaconazole (POS; Schering-Plough, Kenilworth, NJ, USA), isavuconazole (ISA; Basilea Pharmaceutica, Basel, Switzerland), caspofungin (CAS; Merck Sharp & Dohme BV, Haarlem, The Netherlands), anidulafungin (ANI; Pfizer Central Research), and micafungin (MICA; Astellas Pharma, Ibaraki, Japan) were used in this study. The drugs were obtained as reagent grade powders and were preserved according to the manufacturer's instructions.

Table 1
Origin of *Aspergillus flavus* isolates used for in vitro antifungal susceptibility testing

Place	Type	Site	No.
India	Clinical	Allergic fungal rhinosinusitis	77
		Respiratory infections/allergic bronchopulmonary aspergillosis	35
		Invasive fungal rhinosinusitis	28
		Keratitis	30
		Endophthalmitis	3
		Others[a]	5
	Environmental		2
Netherlands	Clinical[b]		18
	Environmental[c]		10
		Total	208

[a] Left maxillary osteomyelitis (n = 1), skin wound (n = 2), renal infarction (n = 1), and frontal cerebral granuloma (n = 1).
[b] Respiratory tract infections (n = 10), invasive fungal rhinosinusitis (n = 2), otitis externa (n = 2), vaginal discharge (n = 2), abdominal abscess (n = 1), allergic fungal rhinosinusitis (n = 1).
[c] Also includes 4 strains of CBS culture collection with different origin.

2.3. Antifungal susceptibility testing

The minimal inhibitory concentrations (MICs) or minimum effective concentrations (MECs) were determined using a broth microdilution method, according to the reference procedure of the Antifungal Susceptibility Testing Subcommittee of EUCAST for spore-forming moulds (EUCAST, 2008; Lass-Flörl et al., 2006, 2008; Perkhofer et al., 2009). Stock solutions (3200 μg/mL) of AMB, ISA, ITR, POS, and VOR were prepared using dimethyl sulfoxide solution. Echinocandins were dissolved in sterile distilled water to a final stock concentration of 3200 μg/mL. Antifungal susceptibility testing was performed in microdilution plates with RPMI 1640 medium with L-glutamine (Difco, Breda, The Netherlands) supplemented with 2% glucose and an inoculum size of 2×10^5 to 5×10^5 CFU/mL. MIC end points were visually determined at 48 h. For polyenes and azoles, the MIC end points were defined as the lowest drug concentration, which resulted in a 100% reduction in growth compared with that of a drug-free growth control. For echinocandins, the MEC was assessed microscopically, corresponding to the lowest drug concentration at which abnormal, short, branched hyphae were observed compared to the long, unbranched hyphae in controls. Minimal fungicidal concentration (MFC) was

determined for AMB and ISA as described previously (Espinel-Ingroff, 2001, Espinel-Ingroff et al., 2002).

Candida parapsilosis (ATCC 22019), *Candida krusei* (ATCC 6258), and *A. flavus* (ATCC 204304) were included in the study as quality control strains.

2.4. Data analysis

MIC_{50} and MIC_{90} were calculated by arranging the MIC data of each antifungal in ascending order and selecting the median and 90th percentile value, respectively. Geometric mean (GM) MICs were computed using Microsoft® Office Excel 2003 SP3. The GM MEC of anidulafungin was calculated by replacing the values "≤X" with "0.5 X". The wild-type (WT) cut-offs were elucidated following the approach used to determine the WT distributions for antibiotics (http://www.srga.org/Eucastwt/bpsetting.htm). This was done by taking the lowest value measured and 3–5 twofold dilutions of the lowest value. WT cut-off was defined when 99% of the strains were inhibited within this distribution. The MIC/MEC results obtained by the EUCAST method were compared to those obtained previously by the CLSI broth dilution method (Shivaprakash et al., 2011). The MIC or MEC value obtained for each isolate by the EUCAST and CLSI method was imported into the SPSS data editor (SPSS 13.0 statistical analysis software for Windows, SPSS, Chicago, IL, USA), and comparison of the means was calculated by applying the Student's *t* test for paired samples. Essential agreement (EA) between the 2 methods was calculated as the percentage of the isolates giving the same results or varying by ±1 log(2) dilutions or by ±2 log(2) dilutions.

3. Results

Table 2 summarizes the in vitro susceptibility of 208 *A. flavus* isolates to all the antifungal agents tested. Fig. 1 shows the MIC distributions and the WT cut-off values of all the antifungal agents against *A. flavus*. For each antifungal tested, the MEC/MIC_{50} and geometric mean MEC/MIC values differed by <1 log(2) dilution, indicating that the MEC/MIC_{50} obtained by inspection reasonably reflects the central tendency of the population. The susceptibility pattern did not show any significant variations among the isolates obtained from different anatomical sites, sources (clinical/environmental), and countries (Netherlands/India).

Except for AMB, all other antifungal agents demonstrated good in vitro activity against *A. flavus*. The MIC of AMB was ≥2 μg/mL for 91.8% of the isolates tested, with 75 (36%) of them exhibiting a MIC ≥8 μg/mL. Among triazoles, the MICs of POS and ITR were ≤0.5 and ≤1 μg/mL, respectively, in all *A. flavus* isolates tested. The MICs of VOR and ISA were ≤2 μg/mL in 95.1% and 98.9% of isolates, respectively. Ten (4.9%) isolates exhibited a MIC of 4 μg/mL for VOR, with 2 of them having a MIC of 4 μg/mL for ISA.

Among the echinocandins, ANI was the most effective, with all the isolates showing a MEC ≤0.016 μg/mL. While the MEC of MICA was ≤0.25 μg/mL in all the isolates tested, the MEC of CAS was ≤0.5 and ≤1 μg/mL in 94.3% and 100% of the isolates, respectively. The MIC distributions were used to determine the WT cut-offs for these agents (Rodriguez-Tudela et al., 2008) (Fig. 1). With the exception of MICA, the WT distributions of all the antifungal agents extended along 2 to 4 log(2) dilutions. The WT distribution of MICA extended beyond 5 log(2) dilutions. Interestingly, the distribution of MICs for AMB was bimodal (Fig. 1A). The MFC of ISA and AMB was 0.4 and 0.5 log(2) dilution higher than the corresponding MIC, respectively.

Essential agreement between the EUCAST method and CLSI method determined as percentage of results within ±1 dilutions was low for AMB (15.5%), ITR (72.2%), POS (80.2%), ISA (89.8%), and MICA (48.6%). When the criterion was increased from ±1 log (2) dilutions to ±2 dilutions, the EA improved (Table 3).

4. Discussion

In the present study, the in vitro susceptibility of 208 *A. flavus* isolates to the new triazole, ISA, was determined by the EUCAST method and compared with other antifungal agents. Except for AMB, all the antifungal drugs tested exhibited a good in vitro activity against the majority of *A. flavus* isolates. In particular, echinocandins demonstrated

Table 2
In vitro susceptibilities of 208 *A. flavus* isolates to isavuconazole and 7 other antifungal agents as determined by EUCAST broth microdilution method

Antifungal agent	MIC/MEC (μg/mL)				Cumulative % of isolates inhibited at MIC (μg/mL) of											
	Range	50%	90%	GM	≤0.008	0.016	0.031	0.062	0.125	0.250	0.500	1.000	2.000	4.000	8.000	16.000
Amphotericin B	1.000–16.000	4.000	8.000	3.52	0	0	0	0	0	0	0	8.2	48.1	64	98.1	100
Itraconazole	0.125–1.000	0.500	0.500	0.37	0	0	0	0	0.5	44.3	98.6	100	100	100	100	100
Voriconazole	0.250–4.000	1.000	2.000	1.31	0	0	0	0	0	0.5	1.9	63.4	95.1	100	100	100
Posaconazole	0.125–0.500	0.250	0.250	0.25	0	0	0	0	3.4	91.9	100	100	100	100	100	100
Isavuconazole	0.250–4.000	1.000	2.000	1.23	0	0	0	0	0	1.4	2.8	66.7	98.9	100	100	100
Caspofungin	0.250–1.000	0.500	0.500	0.47	0	0	0	0	0	13.5	94.3	100	100	100	100	100
Anidulafungin	0.008–0.016	≤0.008	≤0.008	0.004	96.2	100	100	100	100	100	100	100	100	100	100	100
Micafungin	0.008–0.250	0.063	0.125	0.06	0.5	7.2	26.9	64.9	98.6	100	100	100	100	100	100	100

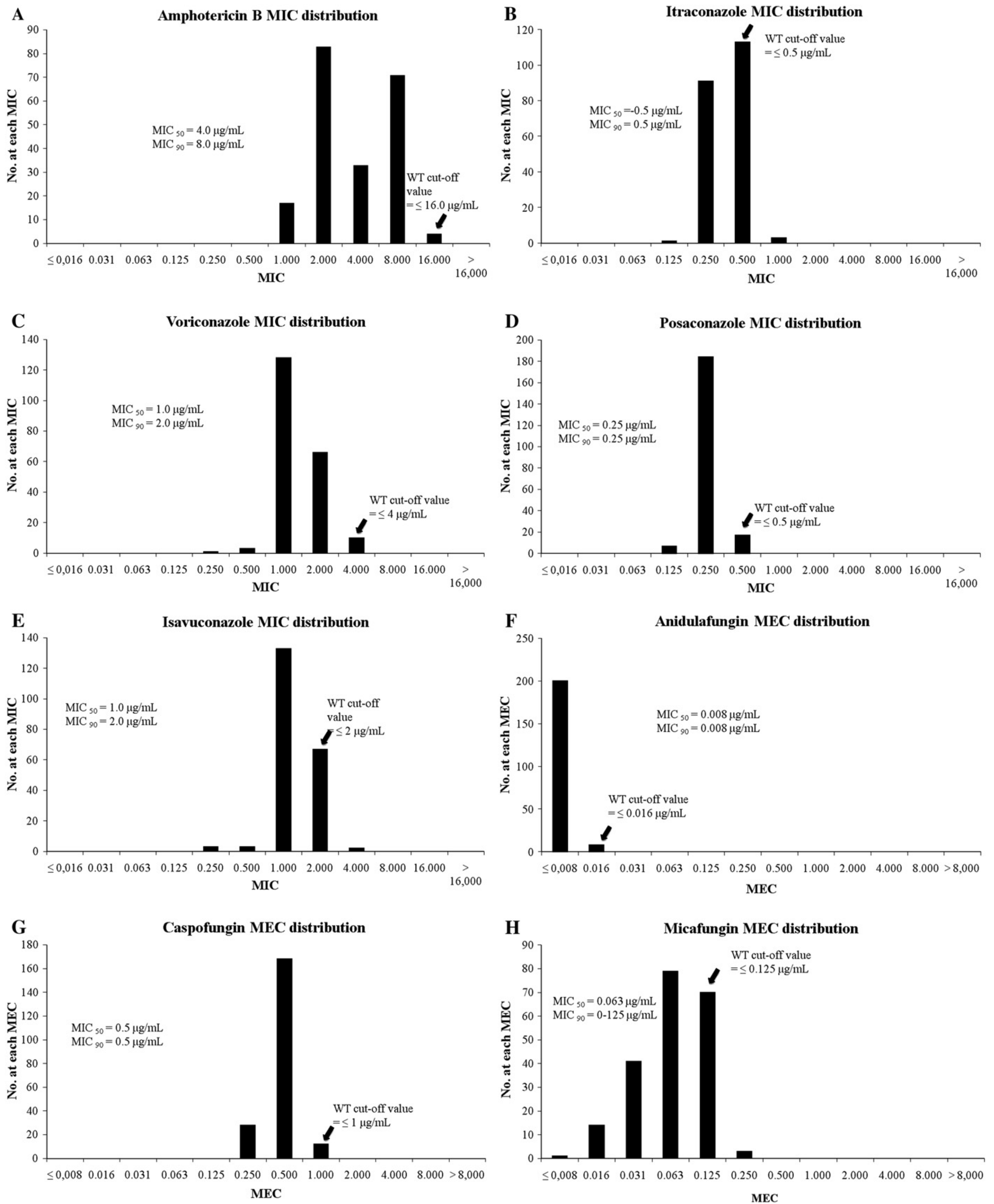

Fig. 1. MIC and MEC distribution of amphotericin B (A), itraconazole (B), voriconazole (C), posaconazole (D), isavuconazole (E), anidulafungin (F), caspofungin (G), and micafungin (H). The arrows indicate suggested WT cut-off values for each drug.

Table 3
Comparison of mean MIC/MECs of 8 antifungals obtained by the EUCAST and CLSI methods (n = 187)[a]

Antifungals		Mean (µg/mL)	Range (µg/mL)	Mean paired differences[a]	95% Confidence interval of the difference[a]		% Essential agreement	
					Lower	Upper	±1 log(2) dilution	±2 log(2) dilutions
Amphotericin B	EUCAST	4.45	1–16	3.81	3.35	4.27	15.5	53.5
	CLSI	0.64	0.25–2					
Itraconazole	EUCAST	0.40	0.25–1	0.21	0.18	0.23	72.2	97.8
	CLSI	0.19	0.062–0.5					
Voriconazole	EUCAST	1.45	0.25–4	0.20	0.10	0.30	98.4	100
	CLSI	1.26	0.5–4					
Posaconazole	EUCAST	0.27	0.125–0.50	0.14	0.13	0.15	80.2	98.3
	CLSI	0.13	0.062–0.25					
Isavuconazole	EUCAST	1.36	0.25–4	0.61	0.54	0.68	89.8	98.9
	CLSI	0.75	0.125–2					
Caspofungin	EUCAST	0.49	0.25–1	−0.02	−0.04	0.01	98.9	99.4
	CLSI	0.51	0.25–1					
Anidulafungin	EUCAST	0.01	0.008–0.016	0.00	0.0003	0.0003	100	100
	CLSI	0.01	0.008–0.016					
Micafungin	EUCAST	0.08	0.008–0.25	0.05	0.04	0.05	48.6	79.1
	CLSI	0.03	0.008–0.125					

[a] Paired sample t test.

excellent activity, showing WT cut-off values similar to those obtained previously by the CLSI broth dilution method (Shivaprakash et al., 2011).

Overall, *A. flavus* was less susceptible to antifungal agents than *A. fumigatus.* These results were comparable to other studies reported for *A. flavus* using the CLSI method (Alcazar-Fuoli et al., 2008; Cuenca-Estrella et al., 2009; Diekema et al., 2003; Sabatelli et al., 2006; Warn et al., 2006). Of specific interest are the results found with AMB. In the present study, the MIC of AMB was ≥2 µg/mL in a large percentage (91.8%) of isolates with a GM MIC of 3.52 µg/mL and a MIC_{90} of 8 µg/mL. The AMB MIC results obtained following the EUCAST guidelines were 2.5 times higher than those determined previously by the CLSI method using almost the same set of isolates (n = 187/188, 1 isolate used in the previous study could not be retrieved and tested by EUCAST) (Shivaprakash et al., 2011). In contrast to the EUCAST, only 1.6% of isolates exhibited a MIC ≥2 µg/mL according to CLSI. The discrepancy between the AMB MIC results obtained by the EUCAST and CLSI method was maximum with EA of 15.5% when the criterion of ±1 dilution was considered and could only improve to 53.5% when this criterion was increased to include ±2 dilutions (Table 3). Such discrepancies between the 2 methods may greatly influence the clinical decision making and treatment especially when the MIC of most of the strains is clustered around the clinical breakpoint. In addition, the bimodal distribution, noticed with the EUCAST method, was not observed with the CLSI technique. Molecular characterization of this collection did not indicate different subspecies (Rudramurthy et al., 2011). Although clinical failure and in vivo resistance to AMB have been reported in occasional strains, AMB still remains the most commonly used antifungal agent for the management of invasive aspergillosis in resource-limited situations (Odds et al., 1998). However, among *Aspergillus* species, MICs of AMB are generally higher for *A. flavus* than for *A. fumigatus* (Sabatelli et al., 2006), possibly due to an alteration of cell-wall composition (Seo et al., 1999).

All the echinocandins demonstrated good in vitro activity against *A. flavus* isolates. ANI was the most effective (MEC_{90} ≤0.008 µg/mL), followed by MICA (MEC_{90} = 0.125 µg/mL) and CAS (MEC_{90} = 0.5 µg/mL). While the WT distributions of MICA were in a broad range, extending over 5 log(2)dilution steps, the values for ANI and CAS were within a narrow range of 2 and 3 log(2) dilutions, respectively. Similar ranges of WT distributions were observed when the same set of isolates was tested by the CLSI method (Shivaprakash et al., 2011). These results support the findings of Rodriguez-Tudela et al. (2010), who reported that glucose concentration (0.2 % CLSI or 2% EUCAST) does not affect the measured MEC. The MEC of echinocandins, obtained in the present study, are consistent with the previous reports of Pfaller et al. (2009, 2010) (for CAS, MICA, and ANI), Warn et al. (2006) (for CAS), Espinel-Ingroff (2003) (for CAS), Oakley et al. (1998) (for CAS), Diekema et al. (2003) (for CAS), and Ruiz-Cendoya et al. (2008) (for MICA). However, Cuenca-Estrella et al. (2009) reported high values (MEC_{50} and MEC_{90} of >16 µg/mL) for all the 3 echinocandins for 81 *A. flavus* strains used in their study. This contrasting result is difficult to explain unless the strains represented different subspecies. Comparison of the present results, obtained by EUCAST, with those observed previously by CLSI (Shivaprakash et al., 2011) for an almost identical set of isolates was good and revealed similar GM MECs of CAS and ANI, but a 1.4 times log(2)

dilution step higher MEC of MICA by EUCAST. Essential agreement of the results of these 2 methods was good for ANI and CAS (100% and 99.4%, respectively, at ±2 dilutions) but less for MICA (79% at ± 2 dilutions). The MEC range and the WT cut-off described for CAS by Pfaller et al. (2010) were lower (0.06 μg/mL) than our results (1 μg/mL). Most (61%) of their isolates were from North and Latin America, and very few (13%) were from the Asia Pacific region. As the majority of isolates in the present study were from India, the differences in susceptibility may be due to differences in the geographical origin.

In a clinical trial, CAS was found to be beneficial in patients with invasive aspergillosis who were refractory to other antifungal agents and could eradicate *A. flavus* more easily than *A. fumigatus* (Denning et al., 2006; Maertens et al., 2004). In a murine model of invasive aspergillosis due to *A. flavus*, ANI was highly effective and the in vivo results correlated well with the in vitro results (Calvo et al., 2011). CAS had been claimed to be highly effective as a first line therapy for the treatment of invasive aspergillosis (Herbrecht et al., 2010); the very low MEC values of ANI suggest it to be also promising for the treatment of invasive aspergillosis caused by *A. flavus.*

Among azoles, POS and ITR demonstrated a more potent in vitro activity as compared to VOR and ISA. These results were in agreement with other studies (Baddley et al., 2009; Cuenca-Estrella et al., 2009; Diekema et al., 2003; Oakley et al., 1998; Perkhofer et al., 2009; Pfaller et al., 2008). Comparison of the results obtained with EUCAST and those with CLSI showed less agreement at ±1 dilution for ITR (72.2%), POS (80.2%), ISA (89.8%), and VOR (EA = 98.4%), but agreement increased to >97% for all these antifungals when the criterion of ±2 dilutions was considered. Similar results (at ±2 dilutions) have been reported by Pfaller et al. (2011), while evaluating ITR, VOR, and POS against *A. flavus*. While the WT MIC cut-off value for ITR and VOR was similar to that of the CLSI method, it was 1 log (2) dilution higher for ISA and POS.

The in vitro activity of ISA was similar to that of VOR, with MIC_{50} and MIC_{90} of 1 and 2 μg/mL, respectively, for the *A. flavus* isolates tested. The MICs of ISA for the *A. flavus* isolates with the EUCAST methodology were found to be higher than that reported in the same group of strains by the CLSI method (Shivaprakash et al., 2011). The present results obtained for ISA conform to the results by Guinea et al. (2008) (GM MIC 0.75 μg/mL) and Warn et al. (2006) (GM MIC 0.73 μg/mL), and were lower than that reported by De La Escalera et al. (2008) (2.41 μg/mL). However, only 12 isolates were tested in the last-named study. For *A. fumigatus*, the breakpoints of VOR and ITR have been suggested to be >2 μg/mL (Verweij et al., 2007). If we consider this breakpoint for *A. flavus*, 10 strains (4.9%) in the present study were resistant to VOR, with a MIC of 4 μg/mL. In comparison, ISA demonstrated better efficacy, with only 2 of those 10 strains exhibiting a MIC of 4 μg/mL. Lionakis et al. (2005) reported that 11% of their *A. flavus* isolates had a high MIC (>2 μg/mL). However, none of the isolates in the present study with EUCAST (98.6% MIC of ≤0.5 μg/mL) and in a previous study with CLSI methods (100% MIC of ≤0.5 μg/mL) demonstrated high MIC for ITR. These results corroborate with the results of many other studies, which revealed a 100% ITR susceptibility among the clinical isolates of *A. flavus* (Baddley et al., 2009; Carrillo-Munoz et al., 2002; Pfaller et al., 2002). The MFCs of ISA and AMB obtained in both the present study and a previous study (Shivaprakash et al., 2011) were only 0.4 and 0.5 log(2) dilution higher than the corresponding MICs, respectively. Lass-Flörl et al. (2003) showed that both the MFC and MIC of AMB against *A. flavus* were the same when tested by CLSI using hyphae.

In conclusion, all antifungal agents tested, except for AMB, showed good in vitro activity against 208 *A. flavus* isolates evaluated in this study. The investigational drug ISA had better activity compared to VOR. In addition, except for AMB and MICA, the results of antifungal susceptibility testing performed with the EUCAST method correlated well with those obtained by the CLSI method reported earlier (Shivaprakash et al., 2011). The results of this study, comprising a large number of strains, suggest the WT cut-off values of each antifungal for *A. flavus*. These may not only serve as a basis for detection of resistance, but also help in suggesting clinical breakpoints for *A. flavus.* However, while considering WT cut-off values, it is important to note that the results obtained in this study were discrepant for AMB, VOR, and ISA by the CLSI and EUCAST methods.

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