A multicenter evaluation of MIC distributions for ECV definition to detect amphotericin B, posaconazole and itraconazole resistance among the most clinically relevant species of Mucorales

A. Espinel-Ingroff,*[1] A. Chakrabarti,[2] A. Chowdhary[3], S. Cordoba,[4] E. Dannaoui,[5] P. Dufresne,[6] A. Fothergill,[7] M. Ghannoum,[8] G. M. Gonzalez,[9] J. Guarro,[10] S. Kidd,[11] C. Lass-Flörl,[12] J.F. Meis,[13] T. Pelaez,[14] A.M. Tortorano,[15] and J. Turnidge[16]

[1]VCU Medical Center, Richmond, VA; [2]Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India; [3]Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; [4]Instituto Nacional de Enfermedades Infecciosas "Dr. C. G. Malbrán", Buenos Aires, Argentina; [5] Université Paris-Descartes; Faculté de Médecine; APHP; Hôpital Européen Georges Pompidou, Unité de Parasitologie-Mycologie, Service de Microbiologie, Paris, France; [6] Institut national de santé publique du Québec, Laboratoire de santé publique du Québec, Sainte-Anne-de-Bellevue, Québec, Canada; [7]University of Texas Health Science Center, San Antonio, TX; [8]University Hospitals Case Medical Center and Case Western Reserve University, Cleveland, OH; [9]Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, México; [10]Facultat de Medicina, IISPV, URV, Reus, Spain; [11]National Mycology Reference Centre, SA Pathology, Adelaide, Australia; [12]The Innsbruck Medical University, Innsbruck, Austria; [13]Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital and Radboud University Medical Center, Nijmegen, The Netherlands; [14]Hospital General Universitario Gregorio Marañón, School of Medicine-Universidad Complutense, Madrid, Spain; [15] Department of Biomedical Sciences for Health,Università degli Studi di Milano, Milano, Italy; [16]University of Adelaide, Adelaide, Australia.

*Corresponding author address:
3804 Dover Rd, Richmond, VA 23221; Phone: (804) 3585895
Email address: avingrof@vcu.edu

Abstract

Clinical breakpoints (CBPs) have not been established for the Mucorales and any antifungal agent. In lieu of CBPs, epidemiologic cutoff values (ECVs) are proposed for amphotericin B, posaconazole and itraconazole and four Mucorales species. Wild type (WT) MIC distributions (organisms in a species/drug combination with no detectable acquired resistance mechanisms) were defined with available pooled CLSI MICs from 14 laboratories (Argentina, Australia, Canada, Europe, India, Mexico, and the United States) as follows: 10 *Apophysomyces variabilis*, 32 *Cunninghamella bertholletiae*, 136 *Lichtheimia corymbifera*, 10 *Mucor indicus*, 123 *M. circinelloides*, 19 *M. ramosissimus*, 349 *Rhizopus arrhizus*, 146 *R. microsporus*, 33 *Rhizomucor pusillus*, and 36 *Syncephalastrum racemosum*. CLSI broth microdilution MICs were aggregated for the analyses. ECVs comprising ≥95% and ≥97.5% of the modeled populations were as follows: amphotericin B ECVs for *L. corymbifera* were 1 and 2 μg/ml, for *M. circinelloides* 1 and 2 μg/ml, for *R. arrhizus* 2 and 4 μg/ml, and for *R. microsporus* 2 and 2 μg/ml, respectively; posaconazole ECVs for *L. corymbifera* were 1 and 2, for *M. circinelloides* 4 and 4, for *R. arrhizus* 1 and 2, and for *R. microsporus* 1 and 2, respectively; both itraconazole ECVs for *R. arrhizus* were 2 μg/ml. ECVs may aid in detecting emerging resistance or those isolates with reduced susceptibility (non-WT-MICs) to the agents evaluated.

Introduction

Although infections caused by filamentous fungi (moulds) are not as prevalent as yeast infections, an increased incidence of systemic infections caused by *Aspergillus* and other mould species and more recently by the Mucorales (Zygomycetes) has been documented (1-3). The order Mucorales comprise a vast variety of genera and species which have been recently reclassified according to DNA barcoding and internal transcribed spacer (ITS) ribosomal sequencing (4). Although most Mucorales species are saprophytic, a large number of these species have been known to cause severe infections (mucormycosis, previously described as zygomycosis), especially among immunocompromised patients and/or patients with granulocytopenia, diabetes and penetrating trauma (5-7). The recommended therapy for

infections caused by the Mucorales is usually surgery and/or one of the amphotericin B lipid formulations; despite its toxicity amphotericin B deoxycholate continues to be used routinely in some areas (5,8). More recently, posaconazole has been recommended as salvage therapy and/or prophylaxis (9-11); itraconazole and other triazoles are also used as prophylactics (9). Despite antifungal therapy, mucormycosis is associated with a great deal of morbidity and about a 50% mortality rate; breakthrough infections caused by Mucorales species are frequently reported among patients receiving triazole prophylaxis, especially voriconazole (3,6).

The Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antifungal Susceptibility Tests has developed a reproducible procedure for the antifungal susceptibility testing of Mucorales species as described in the M38-A2 broth microdilution document (12). However, although species-specific formal breakpoints (CBPs) and/or epidemiological cutoff values (ECVs) have been established for *Candida* spp. and *Aspergillus* spp. (13-16), neither MIC distributions nor ECVs are available for any Mucorales species. The establishment of CBPs for mould species has been hampered by the low incidence of these infections and the scarcity of the data required for their development, including both low and high MICs that might predict clinical failure. However, ECVs are calculated based on MIC distributions (>100 MICs/species/agent) from multiple independent laboratories ($\geq$3) (14,16,17). Although amphotericin B and triazole MIC data have been reported for a variety of genera belonging to the Mucorales, available data are mostly for the more prevalent species and the number of isolates evaluated were small (18-22).

The purpose of the study was (i) to define wild-type-[WT]-susceptibility endpoint MIC distributions of 10 Mucorales species using CLSI M38-A2 broth microdilution MIC data originating from 3 to 14 laboratories and (ii) to propose ECVs for amphotericin B, posaconazole and itraconazole for four common Mucorales species (*Lichtheimia* [*Absidia*] *corymbifera*, *Mucor circinelloides*, *Rhizopus arrhizus* [*R.* oryzae], and *R. microsporus*) when the number of CLSI MICs was $\geq$112 for the species/agent combination originating from $\geq$8 independent laboratories. Amphotericin B, posaconazole and itraconazole MIC distributions comprising 10 to 93 isolates for the less prevalent species (e.g., *Apophysomyces variabilis*, *Cunninghamella bertholletiae*, *Mucor indicus*, *M. ramosissimus*, *Rhizomucor pusillus* and *Syncephalastrum racemosum*) also

are documented. We aggregated a total of 10 to 349 MICs (species and antifungal agent dependent) as obtained in 14 independent laboratories (Argentina, Australia, Canada, Europe, India, Mexico, and the United States).

Materials and Methods

Isolates. The isolates evaluated were recovered from patients with mostly five infections: rhinocerebral, pulmonary, skin, bone, cerebral (some times both cerebral and cutaneous or pulmonary and cutaneous) and abdominal. The most common clinical specimens were: nasal or palate biopsies, aspirates, swabs or scrapes; pulmonary secretions; pleural fluids; CT guided fine needle aspirates; bronchoalveolar lavage and endotracheal aspirates. Antifungal susceptibility testing was performed according to the CLSI broth microdilution method (M38-A2) at the following medical centers: VCU Medical Center, Richmond, VA; Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India; Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; Instituto Nacional de Enfermedades Infecciosas "Dr. C. G. Malbrán", Buenos Aires, Argentina; Institut national de santé publique du Québec, Laboratoire de santé publique du Québec, Sainte-Anne-de-Bellevue, Québec, Canada; University of Texas Health Science Center, San Antonio, TX; University Hospitals of Cleveland and Case Western Reserve University, Cleveland, OH; Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, México; Facultat de Medicina, IISPV, URV**,** Reus, Spain; National Mycology Reference Centre, SA Pathology, Adelaide, Australia; The Innsbruck Medical University, Innsbruck, Austria; Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands; Hospital General Universitario Gregorio Marañón, School of Medicine-Universidad Complutense, Madrid, Spain; and the Università degli Studi di Milano, Milano, Italy. Identification of isolates in each laboratory was performed using molecular methodologies or both conventional and molecular identification (5,7,23). Isolates were not evaluated for either azole or amphotericin B resistance mechanisms. The maximum number of available pooled CLSI MICs from the 14 laboratories for each species was: 10 for *A. variabilis*, 32 for *C. bertholletiae*, 136 for *L. corymbifera*, 10 for *M. indicus*, 123 for *M. circinelloides*, 19 for *M. ramosissimus*, 349 for *R. arrhizus*, 146 for *R. microsporus*, 33 for *Rhizomucor pusillus*, and 36 for *S. racemosum* (Tables

1-3). Although some laboratories submitted separate data for two varieties of *R. microsporus*, ITS sequencing of the varieties of this species has indicated that they are identical (4); therefore, we pooled all these MICs under *R. microsporus* as listed in Tables 1-4. Overall, these isolates represented the unique isolate recovered from each infection and were likely WT strains, but there is no information regarding the prior exposure to antifungal therapy. This could be a possible limitation of the study, as prior exposure may result in acquired antifungal resistance, skewing the results.

Three quality control strains (QC) *Candida parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *Paecilomyces variotii* ATCC MYA-3630, and one reference isolate, *Aspergillus flavus* ATCC 204304, were used by the participant laboratories (12,13).

Antifungal susceptibility testing. In order to include MIC results in the set of aggregated data from the 14 laboratories (Tables 1-3), amphotericin B and triazole MICs were obtained at each center by following the CLSI M38-A2 broth microdilution method (RPMI-1640 broth containing 0.2% dextrose, inoculum concentrations of ~10^4 CFU/ml and 24 h of incubation) (12). The MICs were the lowest drug concentrations that showed 100% growth inhibition or the first clear well as compared to the growth control. At least one or two of the three QC or reference strains were utilized during the years of testing in each center; these MICs were within the recommended MIC limits (13) with one exception. The agreement was 97% for *C. krusei* and amphotericin B (one dilution lower than established range), but the modes were within one dilution.

Definitions. The WT population is the subpopulation of isolates/ MICs in a species/drug combination without detectable acquired resistance mechanisms (17). The ECV is the highest WT susceptibility endpoint; this endpoint has also been defined as the WT cutoff value (CO_{WT}). In other words, the ECV is the critical drug concentration that may identify those strains with decreased susceptibility to the agent being evaluated or the non-WT isolates harboring resistant mechanisms (14,16,17).

Data analysis. The MIC distribution of each species/agent from each laboratory was listed in an Excel spreadsheet; the MIC data were reviewed for obvious outlier results and

abnormalities, e.g., skewed distributions ("truncated"[mode at the lowest concentration tested] or bimodal distributions within an apparent wild-type). These abnormal distributions were not included in the analysis and outliers were not observed. Next, the presumptive WT modal MICs were determined for each species/agent and laboratory followed by obtaining the pooled MIC distributions for each antifungal agent and Mucorales species with the qualifying data. ECVs were calculated for each distribution and species by the previously reported iterative statistical technique (17). Briefly, the modeled population is based on fitting a lognormal distribution to increasing subsets of the data starting at that population that includes isolates with MICs one dilution higher than the mode (or lower mode if more than one mode), and determining the mean and standard deviation of the cumulative lognormal distribution that best fits that data; those numbers were used to calculate the MIC value that captures at least 95% and 97.5% of the modeled WT population (not the observed MIC population). In addition, we evaluated the inherent variability (approximately within one doubling dilution) of susceptibility testing and the presence of outlier laboratories in each pooled distribution (24).

Results and Discussion

For susceptibility testing to be useful in the clinical setting, MIC results should be reliable and must classify the infecting isolate as either resistant (non-treatable) or susceptible (treatable) against the antimicrobial agent being evaluated (25,26). So far, we do not have susceptibility endpoints that would allow such classification for any antifungal agent and species combination belonging to the order Mucorales. The data needed to propose CBPs for these species and any antifungal agent are not available. However, we have gathered sufficient CLSI MICs to propose ECVs of amphotericin B and two triazoles and for four species of Mucorales and to provide MIC distributions for another six less prevalent species. While a total of 15 laboratories submitted MICs of amphotericin B and both triazoles, the distributions for between 1 and 2 laboratories (depending on the antifungal agent and species) were not included in the final analysis due to truncated (modal MIC at the lowest concentration tested) or bimodal ("saddle" between two modes) distributions; itraconazole data were not provided by some laboratories. In addition, several data from one of the laboratories were omitted due to the use of RPMI broth with 2% glucose (rather than 0.2% prescribed by CLSI) (12). Although some of the

laboratories also submitted voriconazole data, most of the modal MICs for the different species were16 μg/ml; the exception was the voriconazole mode of 8 μg/ml for 235 isolates of *R. arrhizus* originating in 11 laboratories (data not shown in Tables 1-3).

The resulting pooled MIC distributions for the three agents and species evaluated as submitted by 3 to 14 laboratories are depicted in Tables 1-3. Evaluation of the pooled MIC distributions indicated that the majority of distributions for each antifungal agent and species were typical for WT organisms (3 to 5 two-fold dilution concentrations surrounding the modal MIC) and that the distributions from each laboratory were comparable as their modal MICs for each species/agent combination were mostly within 1 two-fold dilution of one another. The exceptions were amphotericin B modes for *R. arrhizus* (modes 0.5 to 1 μg/ml in 11 of 12 laboratories, while the mode was 0.25 μg/ml in one laboratory) and itraconazole modes for *L. corymbifera* (modes 0.25 to 0.5 μg/ml in 8 of 9 laboratories, while the mode was 1 μg/ml in one laboratory (data not shown in Tables 1 and 3). The latter modal discrepancy accounts for the three similar "bars" observed in the pooled itraconazole and *L. corymbifera* distribution (Table 3). Amphotericin B modes were species dependent and ranged from 0.06 μg/ml (*S. racemosum*) to 2 μg/ml (*C. bertholletiae*) (Table 1). In contrast to amphotericin B, most posaconazole modes were 0.5 μg/ml; the exceptions were modes of 0.25 μg/ml (*R. pusillus*) and 1 μg/ml (*M. circinelloides* and *A. variabilis*). Physiological, genetic and morphological data have indicated that the most clinically relevant species is *A. variabilis* (27). Data submitted for other two species in this genus (*A. ossiformis* and *A. trapeziformis*) were insufficient to list in Tables 1-3. A wider modal range (0.25 to 4 μg/ml) was observed with itraconazole, as it was for amphotericin B, among the fewer species evaluated, with the lower mode for *L. corymbifera*, *R. pusillus*, and *S. racemosum* and the highest value for *M. circinelloides*, as it was for posaconazole (Tables 2 and 3). Again, some of these distributions are small. On the whole, these results underline the need for identification to the species level as well as for antifungal susceptibility testing.

The *in vitro* activities of the three antifungal agents evaluated are similar to those previously reported for most of the species. In some instances, the pooled amphotericin B MIC ranges were wider for *L. corymbifera*, *M. circinelloides* and *R. pusillus* than previously reported (MIC range for the three species, 0.03-16 μg/ml [Table 1] versus 0.01-0.5 μg/ml) (19, 21,22), but

the number of isolates for these three species was lower (5 to 20 isolates) in those studies and therefore not a good representation of their antifungal susceptibility to amphotericin B. A similar discrepancy in MIC ranges was also observed with the triazole data (21,22), but the most frequent MIC (when provided) was similar to those in the present study. In contrast, in our pooled distributions of *C. bertholletiae* (Tables 1-3), the highest MICs of the three agents ranged between 1 and 8 μg/ml versus reported values of 8 to ≥64 μg/ml for sets of < 7 isolates (19,21). Based on these data and the widespread geographical area from which we have received our MIC data, we surmise that the data are valid.

The CLSI has not made a final decision regarding what ECV percentage (the ≥95% or the ≥97.5% values) to recommend in the future CLSI document under development for this purpose; the lower percentage risks classifying some WT isolates as non-WT isolates, while the higher percentage risks classifying some isolates with acquired resistance mechanisms as WT. Because of that Table 4 depicts amphotericin B, posaconazole and itraconazole ECVs for the aggregated distributions of four species of Mucorales where the data originated in 8 to 14 laboratories and comprised >100 MICs for each species and agent evaluated (using the methodologies that comprised ≥95% and ≥97.5% of the modeled populations). The CLSI amphotericin B ECV comprising ≥95% of the modeled populations is 1 μg/ml for *L. corymbifera* and *M. circinelloides* and 2 μg/ml for *R. arrhizus* and *R. microsporus*; however, ECVs comprising ≥97.5% of the modeled populations were one dilution higher with the exception of *R. microsporus* (both ECVs were 2 μg/ml). It is noteworthy that an amphotericin B MIC of 2 μg/ml is anecdotally considered to be the "breakpoint" for resistance and yet here and among some *Aspergillus* spp. (15) may be perceived as a WT value. The ECV of posaconazole for *L. corymbifera*, *R. arrhizus*, and *R. microsporus* is 1 μg/ml (comprising ≥95% of the modeled populations), while the ECV for *M. circinelloides* is 4 μg/ml. Posaconazole ECVs comprising ≥97.5% of the modeled populations were also one dilution higher, with the exception of the ECV of 4 μg/ml for *M. circinelloides*. Regarding itraconazole, we are proposing a 2 μg/ml ECV for *R. arrhizus*, encompassing both ≥95% and 97.5% of the modeled populations. We did not receive sufficient itraconazole data to propose ECVs for any other species or to propose amphotericin B and posaconazole ECVs for the less prevalent species. Nevertheless, the distributions for the species for which ECVs were not proposed of the three agents are depicted in Tables 1 to 3.

The frequency of amphotericin B and triazole MICs above the ECV (non-WT) varied according to the distribution analyzed (Table 4); it was lower for all species versus amphotericin B (0% to 2.9%) than those for posaconazole (1.8% to 10.9%) (ECVs encompassing ≥95% and ≥97.5% of the MIC populations). As expected, the ≥95% analysis provided the highest rates of non-WT MICs: 2.9% among *L. corymbifera* versus amphotericin B and 10.9% for *R. arrhizus* and posaconazole. Acquired azole resistance in mould isolates has been studied mostly in *Aspergillus* isolates. Targeted disruption of the *cyp51A* gene in azole susceptible *A. fumigatus* isolates has yielded strains with decreased azole susceptibility (MICs > 2 μg/ml) and a reduced concentration of intracellular drug; triazole MICs >4 μg/ml for isolates of *Aspergillus* spp. are associated with clinical failure (28). In a similar manner, the relationship between resistance mechanisms, high amphotericin B MICs and clinical responses to therapy is mostly available for *A. terreus* (intrinsically resistant to this agent), *A. flavus* and some yeast species (29,30). On the other hand, antifungal mechanisms of resistance in the Mucorales are areas that deserve future investigation; to our knowledge no information is available regarding resistance mechanisms of either amphotericin B or posaconazole in these moulds despite the fact that they are the recommended therapeutic agents for mucormycosis. Albeit the prolonged use of amphotericin B, its mechanisms of action and/or resistance are not completely understood, overall, resistance to this agent is considered rare. It is expected that similar mutations could be found among non-WT isolates of the Mucorales versus either amphotericin B or posaconazole as those found in other moulds.

For these moulds and antifungal agents, correlations between MICs and clinical response to therapy were not found in the literature, even though large numbers of mucormycosis cases have been reported. To compound the problem, cultures were not always available since other methods of diagnosis are usually performed to promptly initiate therapy, e.g., histopathology (5,31). Outcome also is influenced by the site of infection, the underlying disease and other factors. However, the correlation of posaconazole MICs and treatment outcome in experimental, disseminated mucormycosis has been evaluated with a variety of Mucorales species. In two of these murine models, immunosuppressed animals infected with either *R. arrhizus* or *R. microsporus* isolates (posaconazole MICs, 2 μg/ml and 0.25 μg/ml for each species), survival

Antimicrobial Agents and Chemotherapy

was higher (30-40 versus 10-20%) when animals were infected with isolates with the lower MICs (32,33). In another study, survival was strain dependent, although posaconazole MICs for both infected strains were low (0.03 and 0.12 μg/ml); though, posaconazole and amphotericin B prolonged survival among neutropenic mice infected with an isolate of *L. corymbifera* for which MICs of both agents were 0.06-1 μg/ml (34). According to our proposed posaconazole ECV for *L. corymbifera* and *R. microsporus*, isolates with the lower MICs and good response to treatment could be considered WT using the values that comprised ≥95% of the modeled populations; the same applies to all *R. arrhizus* infecting isolates (Table 4). Response to posaconazole treatment also has been uncertain in two *M. circinelloides* models; good efficacy was reported when survival was compared to that in non-immunosuppressed control animals, but variable regarding reduction of tissue burden (35,36). The posaconazole ECV for *M. circinelloides* is 4 μg/ml and the isolates evaluated in these two studies could be considered either non-WT (MIC of the infecting isolate, 8 μg/ml) (35) or WT (MICs of the infecting isolates, 1-4 μg/ml) (36). The MIC in the first study was determined at 48 h instead of 24 h. Mechanisms of resistance were not evaluated in any of those strains, because as mentioned above, the molecular biology of the Mucorales is not as developed as that for *Candida* and *Aspergillus*.

In conclusion, we propose species-specific amphotericin B ECVs comprising ≥95% of the modeled populations of 1 μg/ml (*L. corymbifera* and *M. circinelloides*) to 2 μg/ml (*R. arrhizus* and *R. microsporus*); posaconazole ECVs of 1 μg/ml (*L. corymbifera*, *R. arrhizus* and *R. microsporus*) to 4 μg/ml (*M. circinelloides)*; and an itraconazole ECV of 2 μg/ml for *R. arrhizus*. ECVs were mostly one dilution higher using the ≥97.5% of the modeled populations. Our results cover amphotericin B and its lipid formulations because their MIC data have been comparable (29). Further studies should determine the relationship between molecular mechanisms of resistance and our proposed amphotericin B and triazole non-WT values. Although ECVs do not predict clinical response to therapy, they should be considered for inclusion in future CLSI documents under development for ECV setting and use.. Similar to the ECVs for *Candida* spp. and *Aspergillus* spp., the proposed ECVs for the Mucorales may aid in the detection of strains with acquired mechanisms of resistance (non-WT) to the agents evaluated in the present study.

References

1. Gomes MZ, Lewis RE, Kontoyiannis DP. 2011. Mucormycosis caused by unusual mucormycetes, non-*Rhizopus*,-*Mucor*, and -*Lichtheimia* species. Clin. Microbiol. Rev. **24**: 411–445.

2. Kwon-Chung KJ. 2012. Taxonomy of fungi causing mucormycosis and entomophthoramycosis (zygomycosis) and nomenclature of the disease: molecular mycologic perspectives. Clin. Infect. Dis. **54**: S8–S15.

3. Skiada A, Pagano L, Groll A, Zimmerli S, Dupont B, Lagrou K, Lass-Florl C, Bouza E, Klimko N, Gaustad P, Richardson M, Hamal P, Akova M, Meis J F, Rodriguez-Tudela JL, Roilides E, Mitrousia-Ziouva A, Petrikkos G, for the European Confederation of Medical Mycology Working Group on Zygomycosis. 2011. Zygomycosis in Europe: analysis of 230 cases accrued by the registry of the European Confederation of Medical Mycology (ECMM) Working Group on Zygomycosis between 2005 and 2007. Clin. Microbiol. Infect. **17**: 1859–1867.

4. Walther G, Pawłowska J, A. Alastruey-Izquierdo A, Wrzosek M, Rodriguez-Tudela JL, Dolatabadi S, Chakrabarti A, de Hoog GS. 2013. DNA barcoding in *Mucorales*: an inventory of biodiversity. Persoonia. **30**: 11–47.

5. Cornely OA, Arikan-Akdagli S, Dannaoui E, Groll AH, Lagrou K, Chakrabarti A, Lanternier F, Pagano L, Skiada A, Akova M, Arendrup MC, Boekhout T, Chowdhary A, Cuenca-Estrella M, Freiberger T, Guinea J, Guarro J, de Hoog S, Hope W, Johnson E, Kathuria S, Lackner M, Lass-Florl C, Lortholary O, Meis JF, Meletiadis J, Munoz P, Richardson M, Roilides E, Tortorano A. M, Ullmann AJ, van Diepeningen A, Verweij P, Petrikkos G. 2014. ESCMID and ECMM joint clinical guidelines for the diagnosis and management of mucormycosis 2013. Clin. Microbiol. Infect. **20** (Suppl. 3): 5–26.

6. **Ruping MJ, Heinz WJ, Kindo AJ, Rickerts V, Lass-Florl C, Beisel C, Herbrecht CR, Roth Y, Silling G, Ullmann AJ, Borchert K, Egerer G, Maertens J, Maschmeyer G, Simon A, Wattad M, Fischer G, Vehreschild J, Cornely OA**. 2010. Forty-one recent cases of invasive zygomycosis from a global clinical registry. J. Antimicrob. Chemother. **65**: 296–302.

7. Garcia-Hermoso D, Dannaoui E, Lortholary O, Dromer F. 2011. Agents of systemic and subcutaneous mucormycosis and entomophthoromycosis. In: Versalovic J, et al. (Eds.). Manual of Clinical Microbiology, 10th Ed. ASM Press, Washington, DC; 2008-2019.

8. Ostrosky-Zeichner L, **Marr KA, Rex JH, Cohe**n **SH**. 2003. Amphotericin B: time for a new "gold standard". Clin. Infect. Dis. **37**: 415-425.

9. Pagano L, Caira M, Candoni A, Aversa F, Castagnola C, Caramatti C, Cattaneo C, Delia M, De Paolis MR, Blasi RD, Caprio LD, Fanci R, Garzia M, Martino B, Melillo L, Mitra ME, Nadali G, Nosari A, Picardi M, Potenza L, Salutari P, Trecarichi EM, Tumbarello M,

Verga L, Vianelli N, Busca A, for the SEIFEM Group. 2012. Evaluation of the practice of antifungal prophylaxis use in patients with newly diagnosed acute myeloid leukemia: results from the SEIFEM 2010-B registry. Clin. Infect. Dis. **55**: 1515–1521.

10. van Burik JA, Hare RS, Solomon HF, Corrado ML, Kontoyiannis DP. 2006. Posaconazole is effective as salvage therapy in zygomycosis: a retrospective summary of 91 cases. Clin. Infect. Dis. **42**: e61–e65.

11. Vehreschild JJ, Birtel A, Vehreschild MJT, Liss B, Farowski F, Kochanek M, Sieniawski M, Steinbach A, Wahlers K, Fätkenheuer G, Cornely OA. 2013. Mucormycosis treated with posaconazole: review of 96 case reports. Crit. Rev. Microbiol. **39**: 310–324.

12. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, 3rd ed. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Villanova, PA.

13. **Clinical and Laboratory Standards Institute. 2012.** Reference method for broth dilution antifungal susceptibility testing of yeasts; 4th informational supplement. Clinical and Laboratory Standards Institute, Villanova, PA.

14. Espinel-Ingroff A, Pfaller MA, Bustamante B, Canton E, Fothergill A, Fuller J, Gonzalez GM, Lass-Flörl C, Lockhart SR, Martin-Mazuelos E, Meis JF, Melhem MSC, Ostrosky-Zeichner L, Pelaez T, Szeszs MW, St-Germain G, Bonfietti LX, Guarro J, Turnidge J. 2014. Multilaboratory study of epidemiological cutoff values for detection of resistance in eight *Candida* species to fluconazole, posaconazole, and voriconazole. Antimicrob. Agents Chemother. **58**: 2006–2012.

15. Espinel-Ingroff A, Cuenca-Estrella M, Fothergill A, Fuller J, Ghannoum M, Johnson E, Pelaez T, Pfaller MA, Turnidge J. 2011. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). Antimicrob. Agents Chemother. **55**: 5150-5154.

16. **Espinel-Ingroff A, Cuenca-Estrella M, Cantón E.** 2014. EUCAST and CLSI: working together towards a harmonized method for antifungal susceptibility testing. *Curr. Fungal Infect. Rep.* DOI 10.1007/s12281-012-0125-7.

17. Turnidge J, Kahmeter G, Kronvall G. 2006. Statistical characterization of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clin. Microbiol. Infect. **12**: 418-425.

18. Arikan S, Sancak B, Alp S, Hascelik G, McNicholas P. 2008. Comparative in vitro activities of posaconazole, voriconazole, itraconazole, and amphotericin B against *Aspergillus* and *Rhizopus*, and synergy testing for *Rhizopus*. Med. Mycol. **46**: 567–573.

19. Alastruey-Izquierdo A, Castelli MV, Cuesta I, Monzon A, Cuenca-Estrella M, Rodriguez-Tudela J-L. 2009. Activity of posaconazole and other antifungal agents against

Mucorales strains identified by sequencing of internal transcribed spacers. Antimicrob. Agents Chemother. **53**: 1686–1689.

20. Chakrabarti A, Shivaprakash MR, Curfs-Breuker I, Baghela A, Klaassen CH, Meis JF. 2010. *Apophysomyces elegans*: epidemiology, amplified fragment length polymorphism typing, and *in vitro* antifungal susceptibility pattern. J. Clin. Microbiol. **48**: 4580–4585

21. Vitale RG, Sybren de Hoog G, Schwarz P, Dannaoui E, Deng S, Machouart Marie, Voigt K, van de Sande WWJ, Dolatabadi S, Meis JF, Walther G. 2012. Antifungal susceptibility and phylogeny of opportunistic members of the order *Mucorales*. J. Clin. Microbiol. **50:** 66-75.

22. **Alastruey-Izquierdo A, Castelli MV, Cuesta I, Zaragoza O, Monzon A, Mellado E, Rodrıguez-Tudela JL**. 2009. In vitro activity of antifungals against Zygomycetes. Clin. Microbiol. Infect. **15**: 71–76.

23. **Halliday CL, Kidd SE, Sorrell TC, Chen SC-A**. 2014. Molecular diagnostic methods for invasive fungal disease: the horizon draws nearer? Pathology. In press.

24. **Turnidge J, Bordash G.** 2007. Statistical methods for establishing quality control limits in Clinical and Laboratory Standards Institute susceptibility testing. Antimicrob. Agents Chemother. **51**: 2483-2488.

25. Turnidge J, Paterson DL. 2007. Setting and revising antibacterial susceptibility breakpoints. Clin. Microbiol. Rev. **20**: 391-408.

26. Dalhoff A. Ambrose PG, Mouton JW. 2009. A long journey from minimum inhibitory concentration testing to clinically predictive breakpoints: deterministic and probabilistic approaches in deriving breakpoints. Infection. **37**: 296-305.

27. Salas V, Pastor FJ, Calvo E, Sutton DA, Chander J, Mayayo E, Alvarez E, Guarro J. 2012. Efficacy of posaconazole in a murine model of disseminated infection caused by *Apophysomyces variabilis*. J. Antimicrob. Chemother. **67**: 1712-1715.

28. Araujo R, Espinel-Ingroff A. 2010. Antifungal resistance: cellular and molecular mechanisms. In: Ahmad I, et al. (Eds.). Combating Fungal Infections: Problems and Remedy. Springer, London, New York.

29. Blum G, Hörtnagl C, Jukic E, Erbeznik T, Pümpel T, Dietrich H, Nagl M, Speth C, Rambach G, Lass-Flörl C. 2013. New insight into amphotericin B resistance in *Aspergillus terreus*. Antimicrob. Agents Chemother. **57**: 1583-1588.

30. Lestner JM, Howard SJ, Goodwin J, Gregson L, Majithiya J, Walsh TJ, Jensen GM, Hope WW. 2010. Pharmacokinetics and pharmacodynamics of amphotericin B deoxycholate, liposomal amphotericin B, and amphotericinB lipid complex in an *in vitro* model of invasive pulmonary aspergillosis. Antimicrob. Agents Chemother. **54:**3432–3441.

31. Petrikkos G, Skiada A, Drogari-Apiranthitou M. 2014. Epidemiology of mucormycosis in Europe. Clin. Microbiol. Infect. **20**: 67–73

32. Rodríguez MM, Pastor JF, Sutton DA, Calvo E, Fothergill AW, Salas V, Rinaldi MG, Guarro J. 2010. Correlation between *in vitro* activity of posaconazole and *in vivo* efficacy against *Rhizopus oryzae* infection in mice. Antimicrob. Agents Chemother. **54:** 1665–1669.

33. Rodríguez MM, F. Pastor JF, Calvo E, Salas V, Sutton DA, Guarro J. 2009. Correlation of *in vitro* activity, serum levels, and in vivo efficacy of posaconazole against *Rhizopus microsporus* in a murine disseminated infection. Antimicrob. Agents Chemother. **53**: 5022–5025.

34. **Spreghini E, Orlando F, Giannini D, Barchiesi F**. 2010. In vitro and in vivo activities of posaconazole against zygomycetes with various degrees of susceptibility. J. Antimicrob. Chemother. **65**: 2158–2163

35. Sun QN, Najvar LK, Bocanegra R, Loebenberg D, Graybill JR. 2002. In vivo activity of posaconazole against *Mucor* spp. in an Immunosuppressed-mouse model. Antimicrob. Agents Chemother. **46**: 2310–2312.

36. Salas V, Pastor FJ, Calvo E, Alvarez E, Sutton DA, Mayayo E, Fothergill AW, Rinaldi MG, Guarro J. 2012. In vitro and in vivo activities of posaconazole and amphotericin B in a murine invasive infection by *Mucor circinelloides*: poor efficacy of posaconazole. Antimicrob. Agents Chemother. **56**: 2246-2250.

Antimicrobial Agents and Chemotherapy

Table 1. MIC distributions of amphotericin B for 10 Mucorales species from 3 to 13 laboratories, using CLSI M38-A2 microdilution method

Species	No. of isolates tested/labs[a]	MIC (µg/ml) of[b,c]:										
		≤0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32
A. variabilis	10/3	-	1	-	1	-	3	5				
C. bertholletiae	32/6				1	1	5	**16**	8	1		
L. corymbifera	136/12		7	17	36	**53**	19	3	-	-	1	
M. circinelloides	123/13	1	4	14	42	**44**	18	-	-			
M. indicus	10/5	1	-	3	**4**	1	1					
M. ramosissimus	19/5		2	4	3	**6**	1	-	1			
R. arrhizus	257/12	1	3	9	26	64	**112**	39	3			
Rhizomucor pusillus	33/9		2	6	9	**12**	1	2	-	1		
R. microsporus	146/10		2	11	15	**62**	38	15	3			
S. racemosum	35/5	8	**16**	3	6	2						

[a] Number of isolates tested/number of laboratories. [b]Amphotericin B MICs (minimal inhibition concentration) as determined by the CLSI method. [c]Most frequent MIC is bolded.

Table 2. MIC distributions of posaconazole for 10 Mucorales species from 3 to 14 laboratories, using CLSI M38-A2 microdilution method

Species	No. of isolates tested/labs[a]	MIC (μg/ml) of[b,c]:										
		≤0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32
A. variabilis	10/3				1	1	7	1				
C. bertholletiae	30/6				4	**18**	8					
L. corymbifera	112/13		3	9	26	**51**	21	1	1			
M. circinelloides	120/12		2	2	9	21	**49**	26	5	2	4	
M. indicus	10/5				2	**3**	3	1	-	1		
M. ramosissimus	13/4				4	**4**	2	2	1			
R. arrhizus	349/14	1	5	14	80	**154**	57	27	5	-	4	2
Rhizomucor pusillus	33/9		1	4	**10**	10	7	1				
R. microsporus	137/11		3	12	34	**60**	21	4	1	-	2	
S. racemosum	36/5	1	2	4	10	**11**	5	1	2			

[a] Number of isolates tested/number of laboratories. [b]Posaconazole MICs (minimal inhibition concentrations) as determined by the CLSI method. [c]Most frequent MIC is bolded.

Table 3. MIC distributions of itraconazole for 7 Mucorales from 4 to 9 laboratories, using CLSI M38-A2 microdilution method

Species	No. of isolates tested/labs[a]	MIC (μg/ml) of[b,c]:										
		≤0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32
C. bertholletiae	25/4				4	4	**10**	7				
L. corymbifera	93/9		5	10	**24**	21	23	6	3	1		
M. circinelloides	49/8				4	3	7	12	**15**	5	3	
R. arrhizus	215/8		4	9	40	**85**	37	29	2	4	5	
Rhizomucor pusillus	14/5			2	**4**	4	3	1	-	-	-	1
R. microsporus	74/6		1	1	6	15	**25**	20	1	1	4	
S. racemosum	26/5	4	3	5	**7**	4	-	1	-	-	2	

[a] Number of isolates tested/number of laboratories. [b]Itraconazole MICs (minimal inhibition concentrations) as determined by the CLSI method. [c]Most frequent MIC is bolded.

Table 4. Epidemiologic cutoff values (ECVs) for amphotericin B, posaconazole and itraconazole and four Mucorales species as obtained in 8 to 14 laboratories by the CLSI M38-A2 broth microdilution method

Species [a]	Antifungal agent[b]	Range[c]	Mode[c,d,]	Calculated Statistical ECV (% of MICs above the ECV or non-WT)[c,e]	
				≥95%	≥97.5%
L. corymbifera	AMB	0.06-16	0.5	1 (2.9)	2 (0.7)
	POS	0.06-4	0.5	1 (1.8)	2 (0.9)
	ITR	0.06-8	0.25	ND	ND
M. circinelloides	AMB	0.03-4	0.25	1 (0)	2 (0)
	POS	0.06-16	1	4 (5)	4 (5)
	ITR	0.25-16	4	ND	ND
R. arrhizus	AMB	0.03-4	1	2 (1.2)	4 (0)
	POS	0.03-32	0.5	1 (10.9)	2 (3.2)
	ITR	0.06-16	0.5	2 (5.1)	2 (5.1)
R. microsporus	AMB	0.06-4	0.5	2 (2.1)	2 (2.1)
	POS	0.06-16	0.5	1 (5.1)	2 (2.2)
	ITR	0.25-32	1	ND	ND

[a] ECVs only defined for distributions from at least three laboratories using RPMI-1640 as described in the CLSI M38-A2 document (12)
[b] AMB= amphotericin B; POS=posaconazole; ITR=itraconazole
[c] All values expressed in µg/ml.
[d] MIC most frequently obtained for each distribution.
[e] Calculated ECVs comprising ≥95 or ≥97.5 % of the statistically modeled population for each MIC distribution
ND, not determined due to insufficient numbers of laboratories and isolates/species.