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Posaconazole MIC Distributions for *Aspergillus fumigatus* Species Complex by Four Methods: Impact of *cyp51A* Mutations on Estimation of Epidemiological Cutoff Values

A. Espinel-Ingroff,[a] J. Turnidge,[b] A. Alastruey-Izquierdo,[c] E. Dannaoui,[d] G. Garcia-Effron,[e] J. Guinea,[f] S. Kidd,[g] T. Pelaez,[h] M. Sanguinetti,[i] J. Meletiadis,[j] F. Botterel,[k] B. Bustamante,[l] Y.-C. Chen,[m] A. Chakrabarti,[n] A. Chowdhary,[o] E. Chryssanthou,[p] S. Córdoba,[q] G. M. Gonzalez,[r] J. Guarro,[s] E. M. Johnson,[t] J. V. Kus,[u] C. Lass-Flörl,[v] M. J. Linares-Sicilia,[w] E. Martín-Mazuelos,[x] C. E. Negri,[y] M. A. Pfaller,[z] A. M. Tortorano[aa]

[a]VCU Medical Center, Richmond, Virginia, USA

[b]University of Adelaide, Adelaide, Australia

[c]Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

[d]Université Paris-Descartes, Faculté de Médecine, APHP, Hôpital Européen Georges Pompidou, Unité de Parasitologie-Mycologie, Service de Microbiologie, Paris, France

[e]Laboratorio de Micología y Diagnóstico Molecular-Facultad de Bioquímica y Ciencias Biológicas-Universidad Nacional del Litoral, Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), CCT, Santa Fe, Argentina

[f]Servicio de Microbiología Clínica y Enfermedades Infecciosas-VIH, Hospital General Universitario Gregorio Marañon, and Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain

[g]National Mycology Reference Centre, Microbiology & Infectious Diseases, SA Pathology, Adelaide, Australia

[h]Servicio de Microbiología, Hospital Universitario Central de Asturias, Asturias, Spain

[i]Institute of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy

[j]Clinical Microbiology Laboratory, Attikon Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece

[k]Unité de Parasitologie-Mycologie, Département de Virologie, Bactériologie-Hygiène, Parasitologie-Mycologie, DHU VIC, CHU Henri Mondor, Créteil, France

[l]Instituto de Medicina Tropical Alexander von Humboldt-Universidad Peruana Cayetano Heredia, Lima, Peru

[m]Department of Internal Medicine, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan

[n]Department of Medical Microbiology, Postgraduate Institute of Medical Education & Research, Chandigarh, India

[o]Department of Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India

[p]Klinisk Mikrobiologi, Karolinska, Universitetlaboratoriet, Karolinska, Universitetssjukhuset, Stockholm, Sweden

[q]Instituto Nacional de Enfermedades Infecciosas Dr. C. G. Malbrán, Buenos Aires, Argentina

[r]Universidad Autonóma de Nuevo León, Monterrey, Nuevo León, México

[s]Mycology Unit Medical School, Universitat Rovira i Virgili, Reus, Spain

[t]Mycology Reference Laboratory, Public Health England, Bristol, United Kingdom

[u]Public Health Ontario, Toronto, Ontario, Canada

[v]National Mycology Reference Centre, Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria

[w]Universidad de Córdoba, H. G. U. Reina Sofía, Córdoba, Spain

[x]Unidad de Gestión Clínica de Enfermedades Infecciosas y Microbiología, Hospital de Valme, Seville, Spain

[y]Universidade Federal de São Paulo, Laboratório Especial de Micologia, São Paulo, Brazil

[z]University of Iowa College of Medicine, Iowa City, Iowa, USA

[aa]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy

ABSTRACT Estimating epidemiological cutoff endpoints (ECVs/ECOFFS) may be hindered by the overlap of MICs for mutant and nonmutant strains (strains harboring or not harboring mutations, respectively). Posaconazole MIC distributions for the

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Address correspondence to A. Espinel-Ingroff, victoria.ingroff@vcuhealth.org.

Aspergillus fumigatus species complex were collected from 26 laboratories (in Australia, Canada, Europe, India, South and North America, and Taiwan) and published studies. Distributions that fulfilled CLSI criteria were pooled and ECVs were estimated. The sensitivity of three ECV analytical techniques (the ECOFFinder, normalized resistance interpretation [NRI], derivatization methods) to the inclusion of MICs for mutants was examined for three susceptibility testing methods (the CLSI, EUCAST, and Etest methods). The totals of posaconazole MICs for nonmutant isolates (isolates with no known *cyp51A* mutations) and mutant *A. fumigatus* isolates were as follows: by the CLSI method, 2,223 and 274, respectively; by the EUCAST method, 556 and 52, respectively; and by Etest, 1,365 and 29, respectively. MICs for 381 isolates with unknown mutational status were also evaluated with the Sensititre YeastOne system (SYO). We observed an overlap in posaconazole MICs among nonmutants and *cyp51A* mutants. At the commonly chosen percentage of the modeled wild-type population (97.5%), almost all ECVs remained the same when the MICs for nonmutant and mutant distributions were merged: ECOFFinder ECVs, 0.5 μg/ml for the CLSI method and 0.25 μg/ml for the EUCAST method and Etest; NRI ECVs, 0.5 μg/ml for all three methods. However, the ECOFFinder ECV for 95% of the nonmutant population by the CLSI method was 0.25 μg/ml. The tentative ECOFFinder ECV with SYO was 0.06 μg/ml (data from 3/8 laboratories). Derivatization ECVs with or without mutant inclusion were either 0.25 μg/ml (CLSI, EUCAST, Etest) or 0.06 μg/ml (SYO). It appears that ECV analytical techniques may not be vulnerable to overlap between presumptive wild-type isolates and *cyp51A* mutants when up to 11.6% of the estimated wild-type population includes mutants.

KEYWORDS *Aspergillus fumigatus*, CLSI ECVs, ECVs, EUCAST ECVs, Etest, SYO, *cyp51A* mutants, posaconazole, triazole resistance, wild type

Among the species of filamentous fungi (molds), *Aspergillus fumigatus* is the most prevalent species causing severe infections; the attributable mortality rate for aspergillosis is as high as 47%, which is dependent on both the patient population and patient age (1–4). Although *A. fumigatus* frequently affects the lungs and sinuses, *Aspergillus* can infect other organs, including the central nervous system and the heart (4, 5). Posaconazole is recommended as salvage therapy in patients failing first-line treatment for invasive aspergillosis, as well as empirical, prophylactic, and/or adjunctive therapies (5). While routine antifungal susceptibility testing (determination of MICs) is not recommended during initial aspergillosis therapy, susceptibility testing has an important role in identifying potentially resistant isolates, e.g., isolates from patients failing therapy (5). Ideally, MICs ought to be obtained using a reliable antifungal susceptibility assay for which breakpoints (BPs) and/or epidemiological cutoff values (ECVs/ECOFFs) have been established (e.g., by susceptibility testing reference methods). Method-dependent and species-specific ECVs are based on MIC/minimal effective concentration (MEC) data derived from multiple laboratories, and establishment of ECVs is also the first step for establishing breakpoints (6–9). ECVs are particularly important when limited clinical data have precluded the development of BPs, which is the case for many fungal species.

Two reference methods are available for testing the susceptibilities of molds to posaconazole and other agents: the M38 method by the Clinical and Laboratory Standards Institute (CLSI) and a similar microdilution method by the Antifungal Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (10, 11) (http://www.eucast.org/ast_of_fungi/). EUCAST has listed a susceptible BP (0.12 μg/ml) as well as an ECV (ECOFF, 0.25 μg/ml) for posaconazole and *A. fumigatus*. The CLSI has not listed or approved interpretive endpoints for this species/agent (8). A perception has emerged that the suggested posaconazole ECV (either 0.25 or 0.5 μg/ml), which was based on CLSI data from four laboratories, is not suitable in separating nonmutant from mutant isolates, e.g., those harboring *cyp51A* gene mutations (27). An overlap between MICs for presumptive wild-type (WT) and mutant

isolates has recently been documented by EUCAST (http://www.eucast.org/ast_of_fungi/). Other interpretive endpoints (susceptible BP, 0.06 μg/ml; ECV, 0.12 μg/ml; pharmacokinetic/pharmacodynamic [PD] breakpoint, 0.25 μg/ml) have been proposed for posaconazole and *A. fumigatus* using CLSI MICs, PD data, genetic mutations, animal studies, or a combination of these parameters (12, 13).

Among the commercial antifungal susceptibility testing methods (14–16), the broth colorimetric microdilution Sensititre YeastOne (SYO; Trek Diagnostic System, Cleveland, OH) and especially the agar diffusion Etest (bioMérieux, Marcy l'Etoile, France) methods have been evaluated for testing the susceptibilities of molds to posaconazole and other agents (17–19). More recently, these studies have incorporated mutant *A. fumigatus* strains (20–22). However, the testing parameters provided by the manufacturers are more specific for *Candida* spp., and both package inserts list CLSI endpoints as interpretive categories (10, 14, 16). Therefore, there is a need to further investigate these issues by evaluating available posaconazole MICs for the *A. fumigatus* species complex (SC) by these four susceptibility testing methods.

The objectives of the present study were (i) to pool the MICs for isolates of the *A. fumigatus* SC that were obtained by four antifungal susceptibility testing assays (CLSI, EUCAST, Etest, and SYO) and collected from 26 independent worldwide laboratories and published studies (12, 20, 23, 24); (ii) to define method-dependent posaconazole MIC distributions for nonmutant and mutant isolates by each susceptibility testing method; (iii) to examine the suitability of these distributions for each method-dependent ECV setting, including the evaluation of interlaboratory modal agreement; (iv) to evaluate the overlap of MICs for mutant and nonmutant isolates; and (v) to compare the sensitivity of three ECV analytical approaches (the ECOFFinder, normalized resistance interpretation [NRI], and derivatization methods) (9, 25, 26) to the inclusion of MICs for mutant isolates in each nonmutant posaconazole MIC distribution to be analyzed when the distribution comprised >100 MICs that originated in 3 to 15 laboratories. The CLSI MIC distributions for two *Aspergillus* cryptic species (55 *A. lentulus* and 21 *A. udagawae* isolates) collected from three laboratories were also provided. The data for mutants from the participant laboratories originated mostly from European laboratories, in addition to data from Australia, Argentina, and Thailand; by adding data from a published study (24), we also collected data from China.

RESULTS AND DISCUSSION

The recommended major predictor of the clinical response to antimicrobial therapy is the method- and species-dependent BP. In lieu of BPs for mold testing, the CLSI has approved ECVs for various triazoles and species of *Aspergillus* but not for posaconazole and *A. fumigatus* (8, 27). Etest ECVs are available for amphotericin B and the echinocandins and *Aspergillus* isolates (28), but Etest or SYO ECVs for *Aspergillus* spp. and the triazoles have not been proposed. Therefore, we collected the available CLSI, EUCAST, Etest, and SYO posaconazole MICs from 26 laboratories and reevaluated the definition of method-dependent posaconazole ECVs for *A. fumigatus* SC isolates using the CLSI and EUCAST MIC distributions for nonmutant and mutant isolates that originated in 15 and 6 laboratories, respectively, including published studies (12, 23, 24). Using the same methods, we proposed posaconazole Etest and SYO ECVs for *A. fumigatus* SC isolates based on the Etest MIC distributions for nonmutant and mutant isolates and SYO data for nondifferentiated isolates from 8 and 3 laboratories, respectively. The total number of MIC values for mutants determined by the CLSI, EUCAST, and Etest methods originating from published studies versus participant laboratories were as follows: 227 (82.8%) versus 47 (17.2%), respectively, for the CLSI method; 3 (6%) versus 49 (94%), respectively, for the EUCAST method (12, 23, 24); and 5 (17%) versus 24 (83%), respectively, for the Etest method (20) (Table 1). In addition, our ECVs were estimated by the ECOFFinder, NRI, and derivatization procedures to compare their sensitivity to the presence of MICs for mutants within each mixed MIC distribution of nonmutant and mutant isolates. We also examined the overlap between our posaconazole MICs for nonmutant versus mutant isolates of the *A. fumigatus* SC using a substantial number of

TABLE 1 Posaconazole MICs for 355 *Aspergillus fumigatus sensu stricto cyp51A* mutants determined by three susceptibility testing methods[a]

		No. of isolates with MIC (μg/ml) of:										
Mutation[b]	Method	0.016	0.03	0.06	0.125	0.25[c]	0.5	1	2	4	8	≥16	Total
TR34/L98H	CLSI	1	1	0	3	53	109	30	8	1		0	206
	EUCAST					4	10	5	4			1	24
	Etest						1	2	5	1		4	13
G54E/R/V/W	CLSI					1	6	2	5			7	21
	EUCAST						1		1			4	6
	Etest								1	1		3	5
TR46/Y121F	CLSI						1	7	1				9
	EUCAST					1		9					10
	Etest							1					1
M220I/R/T/V/K	CLSI			1		2	3	4	1			2	13
	EUCAST					1		1				1	3
	Etest					1			1		1	1	4
G448S	CLSI				1			5					6
	EUCAST				1	4							5
	Etest					1	1	3					5
G138C	CLSI			1						2	1	1	5
	EUCAST									1			1
	Etest												0
Other[b]	CLSI		2	1	1	1	4	3	1			1	14
	EUCAST		1				1	1					3
	Etest								1				1

[a]MICs were determined by the CLSI M38-A, EUCAST, and Etest methods (10, 11, 16).
[b]Other includes F219I, 1301T, M172, P216L, Y431, TR34/L98H + M172V, and unknown (most commonly, G54E and M220I); mutant data are from the study laboratories and previous studies (12, 20, 23, 24).
[c]The postulated ECV is 0.25 μg/ml.

MICs for mutants (n = 355) determined by three of the four susceptibility testing methods (CLSI, EUCAST, and Etest). To our knowledge, there are no other species/agent combinations with such a large number of MIC data for mutants and nonmutants to test the effectiveness of the different analytical methods.

The criteria for ECV definition have recently been postulated by the CLSI and summarized elsewhere (6, 7, 9). Those criteria were met for the minimum of 100 MIC/MEC values in a pool of data points for ECV definition analysis (Table 2); the minimum number of isolates for an individual nonmutant distribution by the three methods was 24, higher than the acceptable 5 (CLSI) or 15 (EUCAST) (EUCAST Standard Operating Procedure [EUCAST SOP 10.0; http://www.eucast.org/documents/sops/]). The maximum number of isolates in individual distributions before pooling was 449, or 20% of the total 2,223 nonmutant data points, by the CLSI method (Table 2). Thus, there was no need to weigh the distributions used for the analysis, because none of the single distributions included ≥50% of the entire nonmutant population evaluated by three of the four methods (the smallest number of isolates in the pool was 25, or 1%); the exception was a single distribution by the SYO method that included 56% of the data points used to define the tentative SYO ECV.

Among the 2,223 nonmutant isolates for which CLSI MICs were available, 58% (1,289 data points) were *A. fumigatus sensu stricto* and 42% were *A. fumigatus* SC isolates (e.g., identification confirmed by either molecular methods [e.g., matrix-assisted laser desorption ionization–time of flight {MALDI-TOF} mass spectrometry and β-tubulin and calmodulin sequencing] or morphological methods) (29, 30). After pooling of the MICs for nonmutants, there was no observable difference in the MIC distributions between *A. fumigatus* SC and *A. fumigatus sensu stricto* strains. All mutant isolates were *A. fumigatus sensu stricto* (Table 2). Of the four distributions evaluated in a prior study (27),

TABLE 2 Pooled posaconazole MIC distributions for *Aspergillus fumigatus* SC isolates from between 3 and 15 laboratories determined by four susceptibility testing methods[a]

Method and type of MIC distribution[b]	No. of labs	No. of isolates	No. of isolates with MIC (μg/ml) of[c]:										
			≤0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	≥16
CLSI													
Nonmutants	15	2,223	39	332	597	**762**	365	89	26	5	2	2	4
Mutants	6	274	1	3	3	5	57	**123**	51	16	3	1	11
Merged data	15	2,497	40	335	600	**767**	422	212	77	21	5	3	15
EUCAST													
Nonmutants	6	556	7	60	195	**214**	73	7					
Mutants	6	52		1		1	10	12	**16**	5	1	0	6
Merged data	6	608	7	61	195	**215**	83	19	16	5	1	0	6
Etest													
Nonmutants	8	1,365	56	105	529	**572**	75	14	5	6	1	0	2
Mutants	5	29					2	2	6	8	2	1	8
Merged data	8	1,394	56	105	529	**572**	77	16	11	14	3	1	10
SYO	3	381	134	**157**	45	20	11	7	4	2	0	0	1

[a]Posaconazole MICs were obtained by following both the CLSI and EUCAST reference microdilution methods as well as the commercial Etest agar diffusion and SYO broth dilution colorimetric assays (10, 11, 13, 15, 16).
[b]WT, pooled posaconazole MICs for nonmutants; Mutants, pooled posaconazole MICs for isolates harboring *cyp51A* gene mutations; Merged data, aggregated posaconazole MIC distributions for nonmutants and mutants. Among the WT isolates, 58%, 33%, and 29% of the MICs were for *A. fumigatus sensu stricto* isolates by the CLSI, Etest, and SYO methods, respectively. All EUCAST data were for *A. fumigatus sensu stricto* isolates.
[c]The highest number in each row (showing the most frequently obtained MIC or the mode) is indicated in boldface.

the largest was excluded due to an aberrantly low mode (1,152 data points). The analysis of modal variability indicated that of the CLSI posaconazole MICs collected from 18 independent laboratories, 13 had acceptable distributions. These data were pooled with data from two previous studies for further analyses (12, 23); the modes from the 15 laboratories ranged from 0.06 to 0.12 μg/ml, an acceptable distribution pool for ECV definition according to the CLSI criteria for this purpose (7, 8). The excluded distributions from five laboratories were truncated, had no clear mode, or had modes at least 2 dilutions either below (0.016 μg/ml) or above (1 μg/ml) the global mode of 0.12 μg/ml (6, 7). Similar screening has been performed for other CLSI ECVs with comparable exclusion rates; e.g., 4 of 13 distributions were not pooled for the definition of the CLSI ECV for *Candida albicans* versus fluconazole due to aberrant distributions (6). The mode for the merged 274 *A. fumigatus sensu stricto* mutants (47 versus 227 isolates from the study laboratories and previous studies, respectively) was higher, 0.5 μg/ml (12, 23). CLSI posaconazole MICs for the 55 *A. lentulus* isolates ranged from 0.12 to 4 μg/ml (mode, 0.5 μg/ml), and those for the 21 *A. udagawae* isolates ranged from 0.25 to 1 μg/ml (mode, 0.25 μg/ml) (29, 30). Responses to the survey indicated that the CLSI MICs were determined according to the M38-A2 method testing conditions (described below). Overall, MICs for the quality control (QC) isolates were within expected MIC limits (10); the exceptions were that 4.5% of posaconazole MICs for the QC isolates *Candida krusei* ATCC 6258 and *C. parapsilosis* were 1 dilution lower than the expected limits (0.06 to 1 μg/ml and 0.03 to 0.25 μg/ml, respectively). It is noteworthy that CLSI has lowered the posaconazole MIC limit for the QC isolate *C. parapsilosis* ATCC 22019 from 0.06 to 0.25 μg/ml to 0.03 to 0.25 μg/ml (CLSI, minutes of the annual meeting, 8 January 2011, Orlando, FL).

EUCAST posaconazole MICs for 556 nonmutant and 52 mutant *A. fumigatus sensu stricto* isolates from five independent laboratories were pooled and merged with published data (24) (Table 2). The modes for the six individual distributions were comparable, with an overall mode of 0.12 μg/ml, or the same as that for the CLSI data. Therefore, all collected distributions were included for further ECV analysis. The MIC ranges for nonmutant and mutant isolates were slightly more discriminatory by the

EUCAST method than by the CLSI method (≤0.016 to 0.5 μg/ml for nonmutants versus 0.03 to ≥16 μg/ml for mutants). The EUCAST method seemed to provide a better split of the MICs for nonmutants and mutants, with a mode for the mutants of 1 μg/ml versus a CLSI mode of 0.5 μg/ml. There was a noticeable difference between the EUCAST and CLSI wild-type distributions: the two methods had similar means ($\log_2$, −3.94 versus −3.86, respectively) but the EUCAST method had a lower standard deviation (SD) ($\log_2$, 0.897 versus 1.124, respectively) (ECOFFinder analysis). These differences may be due to the smaller number of laboratories and EUCAST MICs in the total.

Etest posaconazole MICs for 1,394 isolates of *A. fumigatus* SC (a total of 450 [33%] of the 1,365 nonmutant isolates and the 29 mutants were *A. fumigatus sensu stricto*) from 7 of 9 independent laboratories were acceptable and were merged with those of a previous study (20) (Table 2). The two excluded distributions were either truncated or had an unacceptable low mode (0.03 μg/ml) 2 dilutions below the global mode of 0.12 μg/ml and the same mode as that for both reference methods. The responses to the survey from each of the nine laboratories revealed that Etest posaconazole MICs were obtained by using solidified RPMI medium supplemented with 2% dextrose and that MICs were determined after 24 h but mostly at 48 h of incubation (when there was an absence of growth in the inhibition ellipse). Again, MICs were outside (4.6%, 1 dilution lower values) the expected limits for the QC isolates *C. parapsilosis* ATCC 22019 (0.03 to 0.25 μg/ml) and *C. krusei* ATCC 6258 (0.12 to 0.25 μg/ml) per the manufacturer's table (16). There was also a difference between the Etest and CLSI nonmutant distributions: the former method had a higher geometric mean ($\log_2$, −4.042 versus −3.86, respectively) and a lower SD ($\log_2$, 0.779 versus 1.124, respectively). These discrepancies could be due to the different susceptibility testing methodologies (broth microdilution versus agar gradient diffusion).

Only 3 of the 8 submitted single SYO posaconazole MIC distributions for 381 *A. fumigatus* SC isolates (29% [110 data points], *A. fumigatus sensu stricto*) were pooled for further ECV analyses. The global modal MIC was 0.03 μg/ml, or much lower than that obtained by the other three susceptibility testing methods (Table 2). The five excluded distributions were mostly truncated or had no obvious mode. Although SYO posaconazole data for mutant isolates of *A. fumigatus* have been documented (21, 22), the nonmutant MIC distributions were not comparable to our pooled MIC distribution. One possible reason for the discrepancy is the fact that different MIC determination criteria and incubation times were utilized in this and previous studies (17, 18, 21, 22). SYO MICs for the QC isolates *C. parapsilosis* ATCC 22019 (0.06 to 0.25 μg/ml) and *C. krusei* ATCC 6258 (0.06 to 0.5 μg/ml) were all within the accepted MIC limits (16). Responses to the surveys indicated that the SYO MICs from these three laboratories were obtained using the basic conditions for this broth colorimetric microdilution assay: a color change from blue to red (instead of growth inhibition) after 48 h of incubation.

Table 3 depicts the ECOFFinder and NRI posaconazole ECVs for 95% and 97.5% of the population (referred to here as 95% and 97.5% ECVs, respectively), as well as the single ECVs by the derivatization method, for the different CLSI, EUCAST, Etest, and SYO MIC distributions for the *A. fumigatus* SC isolates that were evaluated. The ECOFFinder and NRI CLSI 97.5% ECVs were 0.5 μg/ml when the MICs for the mutant and nonmutant distributions were merged. However, the CLSI 95% ECOFFinder ECV was 1 dilution lower (0.25 μg/ml) when the MIC distribution for only nonmutant isolates was analyzed. For the EUCAST and Etest methods, both the 95% and the 97.5% ECOFFinder ECVs were 0.25 μg/ml. Therefore, although the inclusion of EUCAST and Etest MICs for the mutants did not impact the ECV calculation, it impacted the 95% ECOFFinder CLSI result. In our study, that could be due to the fact that ECOFFinder used more data points, while NRI utilized only data from the left-hand side of the bell curve and, obviously, the number of CLSI MICs for mutants was much higher (274 isolates) than that by the EUCAST and Etest methods (52 and 29 isolates, respectively) (Table 2 and Fig. 1). The smaller number of mutants was less likely to modify the ECV. For that reason, although the 97.5% ECVs are the preferred CLSI susceptibility endpoints, the 95% ECOFFinder posaconazole ECV

TABLE 3 ECVs by two analytical techniques for *A. fumigatus* SC isolates based on MICs determined by four susceptibility testing methods and originating from 3 and 15 laboratories

Method and distribution[a]	No. of isolates	ECV calculations for ≥95/≥97.5% of the population by[b]:	
		ECOFFinder	NRI
CLSI			
Nonmutants	2,223	0.25/0.5	0.5/0.5
Merged data	2,497	0.5/0.5	0.5/0.5
EUCAST			
Nonmutants	556	0.25/0.25	0.5/0.5
Merged data	608	0.25/0.25	0.5/0.5
Etest			
Nonmutants	1,365	0.25/0.25	0.5/0.5
Merged data	1,394	0.25/0.25	0.5/0.5
SYO, unknown mutant status	381	0.06/0.06	0.12/0.12

[a]Nonmutants, pooled posaconazole MICs for nonmutant isolates; Merged data, aggregated posaconazole MIC distributions for mutants and nonmutants.

[b]ECVs for ≥95% and ≥97.5% of the statistically modeled population by ECOFFinder and NRI calculations and based on the MICs determined by four susceptibility testing methods (9–11, 13, 15, 16, 25).

of 0.25 μg/ml could be a more useful endpoint for this species/agent combination. Given that only 3 of the 8 available SYO MIC distributions were suitable for ECV analysis, we are proposing a tentative ECOFFinder ECV of 0.06 μg/ml, until more SYO posaconazole data are gathered. The derivatization method also yielded ECVs of 0.25 μg/ml for the different CLSI, EUCAST, and Etest MIC distributions evaluated and an ECV of 0.06 μg/ml for the SYO method. It is noteworthy than an ECV of 0.25 μg/ml was the

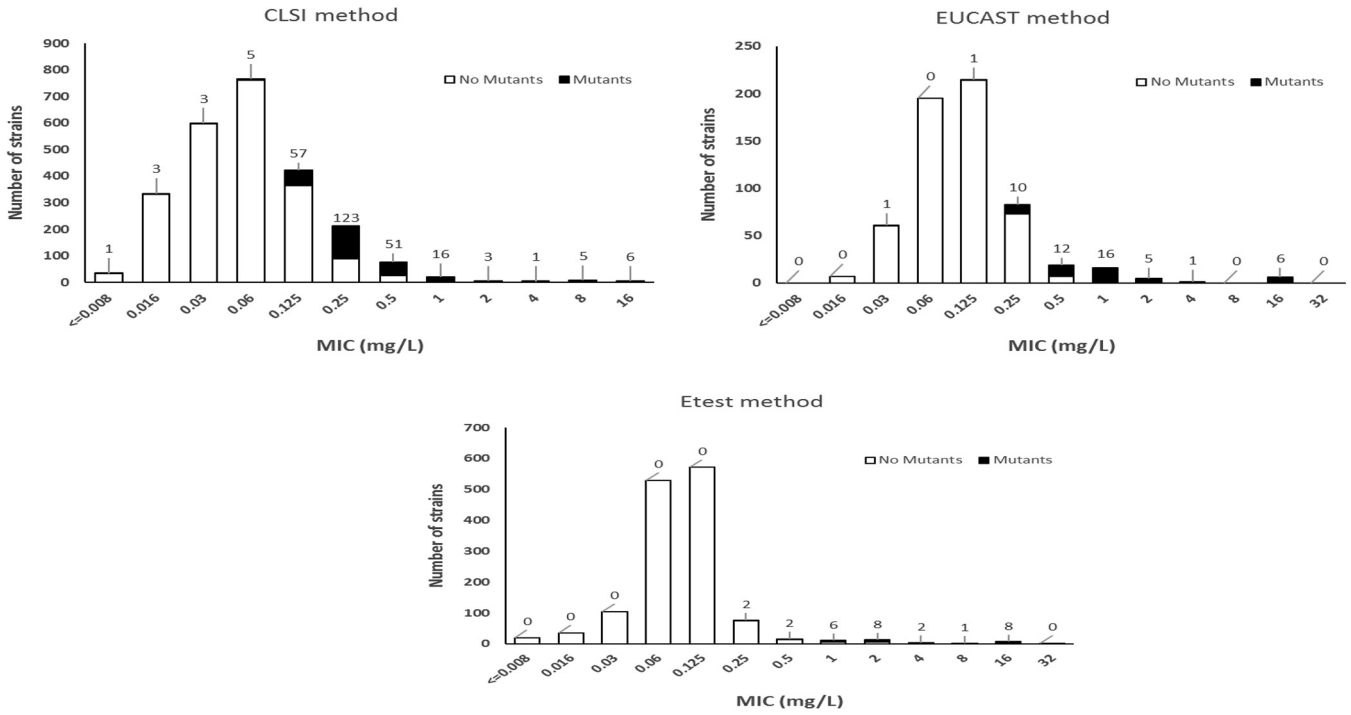

FIG 1 Posaconazole MIC distributions for mutant isolates (harboring *cyp51A* mutations) and nonmutant isolates of the *A. fumigatus* SC by three susceptibility testing methods showing the MIC overlap between both MIC distributions. The number of mutant isolates is indicated above the bar for each MIC.

endpoint previously proposed for the CLSI method (27) and is advocated by EUCAST (http://www.eucast.org/ast_of_fungi/).

The most frequent resistance mechanisms in *A. fumigatus* are modifications in the azole target enzyme CYP51A (30). The primary role of the ECV is to assist the laboratory in identifying isolates with phenotypically expressed acquired resistance mechanisms (6, 7, 9). Given that the ECV does not predict the response to therapy, a "non-WT may or may not respond to therapy" with the agent being evaluated, in this particular case, posaconazole (7). For posaconazole, it is clear that some mutations do not affect the phenotype to the same extent that mutations affect the phenotype for other triazoles; alternatively, it could be that some mutations might actually be simple (silent) polymorphisms (30).

A total of 355 posaconazole MICs for mutant isolates were collected (Table 1 and Fig. 1). The integration of a tandem repeat of 34 bp at the *cyp51A* promoter, along with a mutation that produced the replacement of leucine 98 by a histidine at Cyp51Ap (TR34/L98H), was the most frequent *cyp51A* mutation observed in the strains included in this study (~68%), followed by the amino substitutions at glycine 54 (G54/E/R/W, 9%) or at methionine 220 (M220/I/R, ~6%). The percentage of TR46/Y121F/T289A mutants among the three methods was ~6%. Although most *cyp51A* alterations reduce the phenotype of susceptibility to itraconazole (MICs $>$ 8 μg/ml), there is some selection/specificity regarding their effect on the MICs of other triazoles (30). In our study, we observed an overlap between the MICs for mutant isolates (e.g., isolates linked with the mutations TR34/L98H, G54E, M220/I/T, G448S, G138C, and others) and those for nonmutant isolates, that is, MICs of $\leq$0.25 μg/ml, by the three methods (Tables 1 and 2). A similar overlap was also reported in other studies not only for posaconazole but also for voriconazole and, to a lesser extent, for itraconazole by both reference methods (MIC ranges for *cyp51A* mutants, 0.06 to $>$8 μg/ml), while the MICs for nonmutants could have data points above the ECVs for these three agents (0.06 to $>$8 μg/ml) (12, 31–34).

Another reason for proposing the lower ECOFFinder ECV of 0.25 μg/ml (which was also the same with the derivatization method) is that selecting the lower percentage of the modeled MIC distribution should increase the probability that the ECV would capture a higher proportion of mutants (9). If the objective is to enhance the detection of likely *cyp51A* mutants in particular, then on the basis of the current data, a CLSI-based ECV of 0.5 μg/ml would misclassify 1.8% of nonmutants as non-wild type and 70.1% of mutants as wild type, whereas 5.8% and 25.2% of isolates, respectively, would be so classified if the ECV were set at 0.25 μg/ml. Lowering the ECV even further would increase the likelihood of capturing mutants, but at the risk of greatly increasing the number of wild-type isolates that would be misclassified and subjected to more complex mutation testing.

In conclusion, our abundant aggregated posaconazole MIC data for *A. fumigatus* SC isolates from multiple laboratories and published studies provided a unique opportunity to examine the major overlap in MICs between mutants and nonmutants; it also demonstrated that there is some degree of interlaboratory variability (e.g., aberrant distributions, especially among MICs determined by the SYO method). The CLSI 97.5% ECOFFinder ECV and all NRI endpoints of 0.5 μg/ml are too high if the main aim is to identify isolates with *cyp51A* mutations regardless of their phenotype. The observed overlap between MICs for nonmutant and mutant isolates was more evident with the ECV of 0.5 μg/ml (a higher number of posaconazole MICs of $\leq$0.5 μg/ml for WT isolates). Therefore, although some overlap is still present, the lower posaconazole ECOFFinder ECV of 0.25 μg/ml for the CLSI, EUCAST, and Etest methods could be more clinically relevant; this value has been previously proposed for both reference methods. While we propose a tentative ECOFFinder SYO ECV of 0.06 μg/ml, the evaluation of the SYO MIC distributions from individual laboratories indicated that this method yields less reliable and much lower MICs than those yielded by the reference methods, possibly due to the different MIC determination criteria used by the laboratories. At this stage,

the SYO method should probably not be used for routine testing in the clinical laboratory for this species/agent combination.

MATERIALS AND METHODS

Isolates. The isolates evaluated were recovered from deep infections at sterile and other sites (mostly [>90%] bronchoalveolar lavage fluid, sputum, and other respiratory-related clinical specimens) at the following medical centers: VCU Medical Center, Richmond, VA, USA; Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; Hôpital Européen Georges Pompidou, Paris, France; Laboratorio de Micología y Diagnóstico Molecular-Facultad de Bioquímica y Ciencias Biológicas-Universidad Nacional del Litoral, Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), CCT, Santa Fe, Argentina; Servicio de Microbiología Clínica y Enfermedades Infecciosas-VIH, Hospital General Universitario Gregorio Marañon, and Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain; National Mycology Reference Centre, SA Pathology, Adelaide, Australia; Servicio de Microbiología, Hospital Universitario Central de Asturias, Asturias, Spain; Institute of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy; Département de Bactériologie Virologie Hygiène Mycologie Parasitologie, Créteil, France; Instituto de Medicina Tropical Alexander von Humboldt-Universidad Peruana Cayetano Heredia, Lima, Peru; Department of Medical Microbiology, Postgraduate Institute of Medical Education & Research, Chandigarh, India; Department of Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; Klinisk Mikrobiologi, Karolinska, Universitetlaboratoriet, Karolinska, Universitetssjukhuset, Stockholm, Sweden; Instituto Nacional de Enfermedades Infecciosas Dr. C. G. Malbrán, Buenos Aires, Argentina; Universidad Autonóma de Nuevo León, Monterrey, Nuevo León, México; Mycology Unit Medical School, Universitat Rovira i Virgili, Reus, Spain; Mycology Reference Laboratory, Public Health England, Bristol, United Kingdom; Public Health Ontario, Toronto, Ontario, Canada; National Mycology Reference Centre, Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria; Universidad de Córdoba, H. G. U. Reina Sofía, Córdoba, Spain; Hospital Valme, Seville, Spain; Universidade Federal de São Paulo, Laboratório Especial de Micologia, São Paulo, Brazil; University of Iowa College of Medicine, Iowa City, IA, USA; and the Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy.

Posaconazole MICs were collected for a total of 5,276 *A. fumigatus* complex isolates. The number of nonmutant MICs in each distribution was as follows: CLSI MICs for 2,223 isolates from 13 participant laboratories and two previous studies (12, 23), EUCAST MICs for 556 isolates from 5 participant centers and one prior study (24), Etest MICs for 1,365 isolates from 7 laboratories and one prior study (20), and SYO MICs for 381 isolates from 3 participant laboratories. In addition, we pooled CLSI, EUCAST, and Etest MICs for, respectively, 274, 52, and 29 well-characterized mutant isolates (harboring *cyp51A* gene mechanisms of resistance, e.g., TR34/L98H, TR46/Y121F/T289A, and others) from both participant laboratories and former studies (12, 20, 23, 24). CLSI posaconazole MICs for 55 *A. lentulus* and 21 *A. udagawae* isolates from three laboratories were also collected. The isolates were identified at each medical center by conventional and molecular methodologies that included macro- and microscopic morphology, thermotolerance (incubation at 50°C), MALDI-TOF mass spectrometry, and β-tubulin and calmodulin sequencing (29, 30). Since molecular identification was not performed for all the isolates evaluated in the present study, we listed the nonmutant isolates in Tables 2 and 3 and Fig. 1 as members of the *A. fumigatus* SC. The percentage of *A. fumigatus* SC isolates versus *A. fumigatus sensu stricto* isolates is provided above; most of the mutant isolates were identified in the individual laboratories submitting data at the level of *A. fumigatus sensu stricto*; the exceptions were 10 mutants among the Etest data. Those isolates suspected of harboring *cyp51a* mutations were screened in the individual laboratories submitting data using published protocols (30).

At least one of the quality control (QC) isolates, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, or *Paecilomyces variotii* ATCC MYA-3630, and/or the reference isolates *A. fumigatus* ATCC MYA-3626 and *A. flavus* ATCC MYA-204304 were evaluated by the different methods in each of the participant laboratories (10, 11, 14, 16).

Antifungal susceptibility testing. Posaconazole MICs were obtained by the four antifungal susceptibility testing methods by following the specific testing conditions per the answers to the survey described below (10, 11, 14, 16). The CLSI M38 broth microdilution method was performed with 1×10^4- to 5×10^4-CFU/ml inoculum suspensions and RPMI 1640 medium (0.2% dextrose), and the EUCAST broth microdilution method was performed with 1×10^5- to 5×10^5-CFU/ml inoculum suspensions and RPMI 1640 medium (2% dextrose). MICs were determined by the two reference methods after 48 h of incubation at 35°C (the MIC was determined from the first well that showed complete inhibition of growth or that was optically clear). The Etest MICs were determined per the manufacturer's guidelines, and the MIC was the lowest drug concentration at which the border of the growth-free elliptical inhibition intercepted the scale on the antifungal strip after 24 h and, mostly, 48 h of incubation. The SYO MICs were determined by the manufacturer's guidelines, and the SYO MIC was the concentration in the first blue well after 48 h. Other specific details, including data for the QC isolates, were discussed above.

Definitions. The following definitions have been widely described elsewhere as well as above (6, 7, 28). The ECV is the highest MIC/MEC distribution of the WT population and should be established by using reliable MIC/MEC distributions from at least three laboratories. A non-WT organism usually shows reduced susceptibility to the agent being evaluated compared to that of the WT (no phenotypic resistance) population. In addition to MIC distributions, the ECV calculation takes into account each laboratory distribution mode, the inherent variability of the test (usually within 1 doubling dilution), and the fact that the ECV should encompass 95 to 97% of isolates. Most published ECVs are based on reference MIC distributions, and ECVs based on other methods could be different. We used the same

criteria and requirements for establishing proposed CLSI, EUCAST, Etest, and SYO method-dependent ECVs.

Surveys. As mentioned above, to investigate the possible causes of modal variability, the 26 participant laboratories providing the different sets of MIC data (Table 2) responded to specific parameters for each method. Overall the questions were as follows: (i) was the medium formulation as indicated for each method, (ii) were the MICs always read at the optimal incubation and time for each method, and (iii) what was the growth inhibition criteria used to determine the MICs for each method?

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