Posaconazole MIC distributions for *Aspergillus fumigatus* SC by four methods: Impact of *Cyp51A* mutations on estimation of epidemiological cutoff values (ECVs/ECOFFs)

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ABSTRACT

Estimating epidemiological cutoff endpoints (ECVs/ECOFFS) may be hindered by the overlap of MICs for mutant and non-mutant strains (harboring or not harboring mutations, respectively). Posaconazole MIC distributions for *Aspergillus fumigatus* SC were collected from 26 laboratories (Australia, Canada, Europe, India, South/North America, Taiwan) and published studies. Distributions that fulfilled CLSI criteria were pooled and ECVs were estimated. The sensitivity of three ECV analytical techniques (ECOFFinder, NRI, derivatization) to the inclusion of MICs for mutants was examined for three susceptibility testing methods (CLSI, EUCAST, and Etest®). The totals of posaconazole MICs for non-mutant (no known *cyp51A* mutations) and mutant *A. fumigatus* isolates were: by CLSI, 2,223 and 274; by EUCAST, 556 and 52; by the Etest®, 1,365 and 29 respectively; 381 Sensititre™ YeastOne™ (SYO) MICs with unknown mutational status were also evaluated. We observed an overlap in posaconazole MICs among non-mutant 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 non-mutant and mutant distributions were merged: ECOFFinder ECVs 0.5 µg/ml (CLSI) and 0.25 µg/ml (EUCAST and Etest®); NRI ECVs: 0.5 µg/ml for all three methods. However, the 95% ECOFFinder CLSI ECV for non-mutants was 0.25 µg/ml. The tentative SYO ECOFFinder ECV 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 and *cyp51A* mutants when up to 11.6% of the estimated wild-type population includes mutants.

INTRODUCTION

Among the species of filamentous fungi (moulds), *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 patient population and age (1-4). Although *A. fumigatus* frequently affects the lung 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 ([MICs [minimal inhibitory concentrations]) is not recommended during initial aspergillosis therapy, susceptibility testing has an important role in identifying potentially resistant isolates, e.g., for 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., susceptibility testing reference methods). Method-dependent and species-specific ECVs are based on MIC/MEC data derived from multiple laboratories and are 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 moulds to posaconazole and other agents: the M38-A2 by the Clinical and Laboratory Standards Institute (CLSI) and a similar microdilution method by the Antifungal Subcommittee of 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 the non-mutant from the mutant isolates, e.g., those harboring *cyp51A* gene mutations. An overlap between MICs for presumptive WT and mutant isolates has been recently documented by the EUCAST (http://www.eucast.org/ast_of_fungi/). Other interpretive endpoints (susceptible BP: 0.06 µg/ml; ECV: 0.12 µg/ml; the PK/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 (13,14).

Among the commercial antifungal susceptibility methods (15-17), the broth colorimetric microdilution Sensititre YeastOne (SYO®; Trek Diagnostic System, Cleveland, Ohio) and especially the agar-diffusion Etest® (bioMérieux, Marcy l'Etoile, France) methods have been evaluated for testing the susceptibilities of moulds to posaconazole and other agents (18-20). More recently, these studies have incorporated mutant *A. fumigatus* strains (21-23). However, the testing parameters provided by the manufacturers are more specific for *Candida* spp. and both package inserts list CLSI endpoints as interpretive categories (10,15,17). Therefore, there is a need to further investigate these issues by evaluating available posaconazole MICs for *A. fumigatus* species complex (SC) by these four susceptibility methods.

The objectives of the present study were: (i) to pool the MICs for isolates of *A. fumigatus* SC obtained by four antifungal susceptibility testing assays (CLSI, EUCAST, Etest and SYO) that were collected from 26 independent worldwide laboratories and published studies (13,21,24,25); (ii) to define method-dependent posaconazole MIC distributions for non-mutant and mutant isolates by each susceptibility 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 mutants and non-mutant isolates; and (v) to compare the sensitivity of three ECV analytical approaches (ECOFFinder, NRI [Normalized Resistance Interpretation] and the derivatization method) (9,26,27) to the inclusion of MICs for mutant isolates in each non-mutant 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 also were provided. The mutant data from participant laboratories originated mostly from European laboratories in addition to data from Australia, Argentina, and Thailand; by adding data from a published study (25), we also collected data from China.

RESULTS AND DISCUSSION

The recommended major predictor of clinical response to antimicrobial therapy is the method and species-dependent BP. In lieu of BPs for mould testing, the CLSI has approved

ECVs for various triazoles and species of *Aspergillus*, but not for posaconazole and *A. fumigatus* (8,12). 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 available CLSI, EUCAST, Etest and SYO posaconazole MICs from 26 laboratories and re-evaluated the definition of method-dependent posaconazole ECVs for *A. fumigatus* SC using CLSI and EUCAST MIC distributions for non-mutant and mutant isolates that originated in 15 and 6 laboratories, respectively, including published studies (13,24,25). Using the same methods, we propose posaconazole Etest and SYO ECVs for *A. fumigatus* SC based on Etest MIC distributions for non-mutant and mutant isolates and SYO data for non-differentiated isolates from 8 and 3 laboratories, respectively. The total number of MIC values for mutants by the CLSI, EUCAST and Etest methods originating from published studies versus participant laboratories were: 227 versus 47 respectively (82.8% and 17.2%]; 3 versus 49 (6% and 94%) (13,24,25) and 5 versus 24 (17% and 83%) (21) (Table 3). 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 non-mutant and mutant isolates. We also examined the overlap between our posaconazole MICs for non-mutant versus mutant isolates of *A. fumigatus* SC using a substantial number of MICs for mutants (n=355) by three of the four susceptibility methods (CLSI, EUCAST and Etest). To our knowledge, there are no other species/agent combinations with such large number of MIC data for mutants and non-mutants to test the effectiveness of the different analytical methods:

The criteria for ECV definition have been recently 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 1); the minimum number of isolates for an individual non-mutant 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 non-mutant data points by the CLSI method (Table 1). Thus, there was no need to weigh the distributions used for the analysis, because none of the single distributions included $\geq$ 50% of the entire non-mutant 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 non-mutant isolates for which CLSI MICs were available, 58% (1,289 data points) were *A. fumigatus sensu stricto* (SS) and 42% *A. fumigatus* SC isolates (e.g., identification confirmed by either molecular [e.g., MALDI-TOF and β-tubulin and calmodulin sequencing] and/or morphological methods) (29,30). After pooling of non-mutants, there was no observable difference in the MIC distributions between SC and SS strains. All mutant isolates were *A. fumigatus SS* (Table 1). Of the four distributions evaluated in the prior study (12), 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 were pooled with data from two previous studies for further analyses (13,24); the modes from the 15 laboratories ranged between 0.06 and 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 two 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* SS mutants (47 versus 227 isolates, study laboratories and previous studies, respectively) was higher, 0.5 µg/ml (13,24). CLSI posaconazole MICs for the 55 *A. lentulus* isolates ranged between 0.12 to 4 µg/ml (mode 0.5 µg/ml) and for the 21 *A. udagawae* between 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 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 *C. krusei* ATCC 6258 and *C. parapsilosis* were one dilution lower than the expected limits (0.06-1 µg/ml and 0.03-0.25 µg/ml, respectively). It is noteworthy that the CLSI has lowered the posaconazole MIC limit for the QC isolate *C. parapsilosis* ATCC 22019 from 0.06-0.25 to 0.03-0.25 µg/ml (CLSI, minutes of the annual meeting, 1/8/2011, Orlando, Fla).

EUCAST posaconazole MICs for 556 non-mutant and 52 mutant *A. fumigatus* SS isolates were pooled from five independent laboratories and merged with published data (25) (Table 1). 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 non-mutant and mutant isolates were slightly more discriminatory by the EUCAST than by the CLSI method (non-mutant $\leq$0.016 to

0.5 µg/ml versus mutant 0.03 to ≥16 µg/ml). The EUCAST method seemed to provide a better split of the MICs for non-mutant and mutants, with a mode for the mutants of 1 µg/ml versus the CLSI mode of 0.5 µg/ml. There was a noticeable difference between the EUCAST and CLSI wild-type distributions: similar mean (log_2: -3.94 versus -3.86, respectively), but a lower standard deviation (log_2: 0.897 versus 1.124, ECOFFinder analysis) by the EUCAST method. 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 1365 non-mutant isolates and the 29 mutants were *A. fumigatus SS*) were acceptable from 7 of 9 independent laboratories and were merged with those of a previous study (21) (Table 1). The two excluded distributions were either truncated or had an unacceptable low mode (0.03 µg/ml), two 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 (absence of growth in the inhibition ellipse). Again, MICs were outside (4.6%, one dilution lower values) the expected limits for both QC isolates *C. parapsilosis* ATCC 22019 (0.03-0.25 µg/ml) and *C. krusei* ATCC 6258 (0.12-0.25 µg/ml) as per the manufacturer's table (17). There was also a difference between the Etest and CLSI non-mutant distributions: the former method had, a higher geometric mean (log_2: -4.042 versus -3.86) and a lower SD (log_2: 0.779 versus 1.124). These discrepancies could be due to the different susceptibility 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* SS) were pooled for further ECV analyses. The global modal MIC was 0.03 µg/ml or much lower than by the other three susceptibility methods (Table 1). 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 (22,23), the non-mutant 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 have been utilized in this and previous studies (18,19,22,23). SYO MICs for the QC isolates *C. parapsilosis* ATCC 22019 (0.06-0.25 µg/ml) and *C. krusei* ATCC 6258 (0.06-0.5 µg/ml) were all within the accepted MIC limits (17). 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: color change from blue to red (instead of growth inhibition) after 48 h of incubation.

Table 2 depicts the ECOFFinder and NRI 95% and 97.5% posaconazole ECVs, as well as the single ECVs by the derivatization method for the different CLSI, EUCAST, Etest and SYO MIC distributions for *A. fumigatus* SC that were evaluated. The ECOFFinder and NRI CLSI 97.5% ECVs were 0.5 µg/ml when the MICs for mutant and non-mutant distributions were merged. However, the CLSI 95% ECOFFinder ECV was one dilution lower (0.25 µg/ml) when the MIC distribution for only non-mutant isolates was analyzed. For the EUCAST and Etest methods, both 95 and 97.5% ECOFFinder ECVs were 0.25 µg/ml. Therefore, although the inclusion of EUCAST and Etest MICs for 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 the ECOFFinder used more data points, while the NRI only utilizes the left-hand side of the bell curve and, obviously, the number of CLSI MICs for mutants was much higher (274) than those by the EUCAST and Etest (52 and 29, respectively) (Table 1 and Figure 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 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 endpoint previously proposed for the CLSI method (12), and is advocated by the EUCAST. (http://www.eucast.org/ast_of_fungi/).

The most frequent resistance mechanisms in *A. fumigatus* are the 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 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 as that of 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 3 and Fig. 1). The integration of a tandem repeat of 34pb at the *cyp51A* promoter, along with a mutation that produced the substitution of the leucine 98 for a histidine at the 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 susceptibility phenotype to itraconazole (MICs >8 µg/ml), there is some selection/specificity regarding their effect on the other triazole MICs (30). In our study, we observed an overlap between MICs for mutant (e.g., isolates linked with the following mutations: TR34/L98H, G54E, M220/I/T, G448S, G138C and others) and non-mutant isolates, that is MICs ≤0.25 µg/ml by the three methods (Table 1 and Table 3). A similar overlap is also reported in other studies, not only for posaconazole, but with voriconazole and to a lesser amount with itraconazole by both reference methods (MIC ranges for *cyp51A* mutants: 0.06->8 µg/ml), while MICs for non-mutants could have data points above the ECVs for these three agents (0.06->8 µg/ml) (13,31-34).

Another reason for proposing the lower ECOFFinder ECVs of 0.25 µg/ml (also the same with the derivatization method) is that selecting the lower percentage of the modelled 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 based on the current data, a CLSI-based ECV of 0.5 µg/ml would misclassify 1.8% of non-mutants as non-wild type, and 70.1% of mutants as wild type, compared to 5.8% and 25.2% respectively if the ECV is 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 from multiple laboratories and published studies provided a unique opportunity to examine the major overlap in MICs between mutants and non-mutants; 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 non-mutant and mutant isolates was more evident with the ECVs of 0.5 µg/ml (higher number of posaconazole MICs ≤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 CLSI, EUCAST and Etest methods could be more clinically relevant; this value has been previously proposed for both reference methods. While we are proposing 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 by the reference methods, possibly due to 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, sterile and other sites (mostly [>90%] bronchoalveolar lavage fluids, 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, UK; Public Health Ontario, 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, Iowa, 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 non-mutant MICs in each distribution were as follows: CLSI MICs for 2,223 isolates from 13 participant laboratories and two previous studies (13,24); EUCAST MICs for 556 isolates from five participant centers and one prior study (25); Etest MICs for 1,365 isolates from 7 laboratories and one prior study (21) and SYO MICs for 381 isolates from three participant laboratories. In addition, we pooled CLSI, EUCAST and Etest MICs for 274, 52, and 29 (respectively) 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) (13,21,24,25). CLSI posaconazole MICs also were collected for 55 *A. lentulus* and 21 *A*. *udagawae* isolates from three laboratories. 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 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 non-mutant isolates in Tables 1, and 2 and Figure 1 as *A. fumigatus* SC. The percentage of *A. fumigatus* SC versus *A. fumigatus* SS is provided above; most of the mutant isolates were identified in the individual laboratories submitting data at the level of *A. fumigatus* SS; 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 following quality control (QC) isolates: *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *Paecilomyces variottii* ATCC MYA-3630 and/or 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 (10,11,15,17).

Antifungal susceptibility testing. Posaconazole MICs were obtained by the four antifungal susceptibility methods by following the specific testing conditions as per answers to the survey described below (10,11,15,17): the CLSI M38-A2 broth microdilution method (1–5 × 10^4 CFU/ml inoculum suspensions, RPMI 1640 medium [0.2% dextrose]) and the EUCAST

broth microdilution method (1–5 x 10^5 CFU/ml inoculum suspensions, RPMI 1640 medium [2% dextrose]). MICs by the two reference methods were determined after 48 h of incubation at 35ºC (first well showing complete inhibition of growth or optically clear). The Etest MICs were determined as per 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 and mostly 48 h of incubation and the SYO MICs by the manufacturer's guidelines, the SYO MIC was the first blue well after 48 h. Other specific details, including data for QC isolates, have been 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 is 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 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 one doubling dilution), and 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 1) responded to specific parameters for each method. Overall the questions were: (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 MICs for each method?

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Antimicrobial Agents and Chemotherapy

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Table 1. Pooled posaconazole MIC distributions of *Aspergillus fumigatus* SC from between 3 and 15 laboratories determined by four susceptibility methods[1].

Type of MIC distribution and method[3,4]	No. labs	No. isolates	No. of isolates with MIC (µg/ml) of:[2]										
			≤0.016	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
CLSI													
Non-mutants	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	2497	40	335	600	**767**	422	212	77	21	5	3	15
EUCAST													
Non-mutants	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													
Non-mutants	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

[1]Posaconazole MICs were obtained by following both CLSI and EUCAST reference microdilution methods as well as the commercial Etest agar diffusion and SYO broth dilution colorimetric assays (10,11,14,16,17).
[2]The highest number in each row (showing the most frequently obtained MIC or the mode) is indicated in boldface.
[3]WT: Pooled posaconazole MICs for non-mutants; Mutants: pooled posaconazole MICs for isolates harboring *cyp51A* gene mutations; Merged data: aggregated posaconazole MIC distributions for non-mutants and mutants.
[4]Among the WT isolates, 58%, 33% and 29% MICs were for *A. fumigatus sensu stricto* (SS) by the CLSI, Etest and SYO methods, respectively. All EUCAST data were for *A. fumigatus* SS isolates.

Table 2. ECVs by two analytical techniques for *A. fumigatus SC* based on MICs determined by four susceptibility testing methods and originating from 3 and 15 laboratories.

Distribution and method[1]	No. isolates	ECV calculations by:[2] ECOFFinder ≥ 95/97.5%	ECV calculations by:[2] NRI ≥ 95/97.5%
CLSI			
Non-mutants	2,223	0.25/0.5	0.5/0.5
Merged data	2,497	0.5/0.5	0.5/0.5
EUCAST			
Non-mutants	556	0.25/0.25	0.5/0.5
Merged data	608	0.25/0.25	0.5/0.5
Etest			
Non-mutants	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

[1]WT: Pooled posaconazole MICs for non-mutant isolates; Merged data: aggregated posaconazole MIC distributions for mutants and non-mutants.
[2]ECVs comprising ≥95% and ≥ 97.5% of the statistically modeled population by ECOFFinder and NRI calculations and based on MICs determined by four susceptibility methods (9-11,14,16,17,26).

Table 3. Posaconazole MICs for 355 *Aspergillus fumigatus* SS *cyp51* Mutants as Determined by Three Susceptibility Methods[1]

Mutation[2]	Method	MIC (µg/mL)											
		0.016	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>=16**	**Totals**
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/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/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[2]	CLSI		2	1	1	1	4	3	1			1	14
	EUCAST		1				1	1					3
	Etest								1				1

[1]MICs determined by the CLSI M38-A, EUCAST and Etest methods (10,11,17). The postulated ECV is 0.25 µg/ml

[2]Includes F219I, 1301T, M172, P216L, Y431, TR34/L98H+M172V, unknown; (most common G54E, M220I); mutant data from study laboratories and previous studies (13,21,24,25)

Antimicrobial Agents and Chemotherapy

Fig. 1. Posaconazole MIC distributions for mutant (clear section of the bar) and non-mutant isolates of *A. fumigatus* SC (isolates harboring *cyp51A* mutations bold section of the bar) by three susceptibility methods showing the MIC overlap between both MIC distributions. The number of non-mutant isolates by each MIC concentration is above the corresponding bar.