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In Vitro Susceptibility Testing of *Aspergillus* spp.: Comparison of Etest and Reference Microdilution Methods for Determining Voriconazole and Itraconazole MICs

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The performance of the Etest for voriconazole and for itraconazole susceptibility testing of 376 isolates of *Aspergillus* spp. was assessed in comparison with the National Committee for Clinical Laboratory Standards (NCCLS) proposed standard microdilution broth method. The NCCLS method employed RPMI 1640 broth medium, and MICs were read after incubation for 48 h at 35°C. Etest MICs were determined with RPMI agar containing 2% glucose and were read after incubation for 48 h at 35°C. The isolates included *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. versicolor*, *A. glaucus*, *A. nidulans*, *A. ustus*, and *A. sydowii*. Overall agreement percentages between the Etest and microdilution MICs were 97.6% for voriconazole and 95.8% for itraconazole. Where a discrepancy was observed between Etest and the reference method, the Etest tended to give lower values with voriconazole and higher values with itraconazole. The Etest method using RPMI agar appears to be a useful method for determining the voriconazole and itraconazole susceptibilities of *Aspergillus* spp.

Aspergillus is arguably the most important of the invasive mycoses among patients with neutropenia and prolonged immunosuppression (2, 12, 14). Invasive aspergillosis occurs at an annual rate of 12.4 infections per million persons, accounting for over 10,000 cases and $548 million in expenses per year in the United States (4, 26). Given the increasing rate of infection and a case fatality rate ranging from 60 to 80%, it is clear that the diagnosis and treatment of invasive aspergillosis is one of the unmet needs in medicine today (14, 17).

Despite the development of lipid formulations of amphotericin B and the approval of intravenous itraconazole (12, 17, 18), antifungal treatment of invasive aspergillosis is far from optimal (14). The recent introductions of voriconazole, for the primary treatment of invasive aspergillosis, and of caspofungin, for salvage therapy, provide new options and new hope to bear in efforts to improve the treatment of this devastating infection (11, 12, 14, 18, 19).

The increase in treatment options for invasive aspergillosis, coupled with documented resistance to amphotericin B and itraconazole among some strains and species (3, 5, 13, 19, 27, 29), has created a need for standardized methods for determining the in vitro susceptibilities of both new and established antifungal agents against clinical isolates of *Aspergillus* species (10, 15, 22). In response to these needs, the National Committee for Clinical Laboratory Standards (NCCLS) has developed a standard broth microdilution (BMD) procedure for antifungal susceptibility testing of *Aspergillus* spp. and other rapidly growing molds (16). This method has proven to be very reproducible and useful in both clinical testing and antifungal surveillance studies (9, 25). With the establishment of this reference method, there is now an opportunity to validate alternative methods for testing molds against the various systemically active antifungal agents (10, 15, 22).

The agar-based Etest MIC method (AB Biodisk, Solna, Sweden) has proven to be useful in testing *Candida* spp. against a variety of antifungal agents, including amphotericin B, flucytosine, the azole antifungal agents, and caspofungin (1, 7, 20–24). This methodology has also been shown in a limited number of studies to be applicable for testing *Aspergillus* spp. and other filamentous fungi (1, 6, 10, 15, 22, 28). Recently, Espinel-Ingroff and Rezusta (10) reported very good agreement between Etest and the NCCLS M38-P reference method in testing 107 isolates of *Aspergillus* spp. against voriconazole, posaconazole, and itraconazole.

The purpose of the present study was to expand the Etest database for voriconazole and itraconazole by testing an international collection of 376 clinical isolates of *Aspergillus* spp. obtained from 34 different locations in eight nations during the period from 2000 to 2001. The voriconazole and itraconazole MIC results determined by the Etest were compared to MICs determined by the NCCLS-proposed reference BMD method NCCLS M38-A (16).

MATERIALS AND METHODS

Organisms. A total of 376 isolates of *Aspergillus* spp. obtained from 34 different medical centers in North America (23 centers), Europe (10 centers), and the Asia-Pacific region (1 center) between January 2000 and December 2001 were tested. The isolates were obtained from a variety of sources including sputum, bronchoscopy, and tissue biopsy specimens. The collection of isolates included 277 *A. fumigatus*, 32 *A. flavus*, 27 *A. niger*, 19 *A. terreus*, 13 *A. versicolor*, 3 *A. nidulans*, 2 *A. ustus*, 2 *A. sydowii*, and 1 *A. glaucus*. All isolates were stored as spore suspensions in sterile distilled water at room temperature until they were used in the study. Before testing, each isolate was subcultured at least twice on potato dextrose agar (Remel, Lenexa, Kans.) to ensure viability and purity.

Etest method. Etest was performed in accordance with the manufacturer's instructions as described previously (22). Isolates were grown on potato dextrose agar slants (Remel) at 35°C for 7 days to ensure adequate sporulation. Spore suspensions were prepared in sterile saline and adjusted to a concentration of 10^6

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spores/ml, corresponding to 80 to 82% transmittance at 530 nm (16, 22). The agar formulation used for the Etest was RPMI 1640 supplemented with 1.5% agar and 2% glucose and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Remel). The 150-mm-diameter plates contained RPMI agar at a depth of 4.0 mm. The plates were inoculated by dipping a sterile swab into the spore suspension and streaking it across the surface of the agar in three directions. The plates were dried at ambient temperature for 15 min before applying voriconazole and itraconazole Etest strips. The plates were incubated at 35°C and read at 48 h. The Etest MIC was read as the drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip.

Reference BMD method. The BMD method was performed according to NCCLS-approved guidelines (16) and as described by Espinel-Ingroff et al. (8, 9). Voriconazole was obtained from Pfizer Pharmaceuticals (New York, N.Y.), and itraconazole was obtained from Janssen Research Foundation (Beerse, Belgium). Stock solutions were prepared in 100% dimethyl sulfoxide (Sigma, St. Louis, Mo.), diluted to 100 times the final concentrations in dimethyl sulfoxide, further diluted in RPMI 1640 medium buffered to pH 7.0 with MOPS buffer, and dispensed into 96-well microdilution trays. Trays containing a 0.1-ml aliquot in each well of the appropriate drug solution (2× final concentration) were subjected to quality control (QC) and then sealed and stored at −70°C until used in the study. The final concentration of voriconazole and itraconazole in the wells ranged from 0.007 to 8.0 μg/ml. The stock conidial suspension (10^6 spores/ml) was diluted to a final inoculum concentration of 0.4×10^4 to 5×10^4 CFU/ml and dispensed into the microdilution wells. The inoculated microdilution trays were incubated at 35°C and read at 48 h. The MIC endpoint for both voriconazole and itraconazole was defined as the lowest concentration that produced complete inhibition of growth (9).

QC. QC was ensured by testing the following strains recommended in the NCCLS M38-A approved standard (16): *A. flavus* ATCC 204304, *Candida parapsilosis* ATCC 22019, and *Candida krusei* ATCC 6258. All results were within the recommended limits.

Analysis of results. Etest MICs read at 48 h were compared to reference microdilution MICs read at 48 h. The reference microdilution MICs and Etest MICs were determined in two different time periods and were read independently by two different individuals; i.e., the testing was blinded. Since the Etest scale has a continuous gradient of concentrations, the MICs in between twofold dilutions were rounded to the next twofold level of the reference method for comparison (10, 22). Off-scale MICs at the upper limit were converted to the next higher concentration, and off-scale results at the lower end were left unchanged. Discrepancies between the MICs of no more than 2 dilutions were used to calculate the percent agreement.

RESULTS AND DISCUSSION

Table 1 summarizes the in vitro susceptibilities of 376 clinical isolates of *Aspergillus* spp. to voriconazole and itraconazole as determined by the reference microdilution and Etest methods. The data are presented in a continuous fashion as the cumulative percentages of organisms susceptible at each dilution throughout the dilution series. The MIC results obtained by both the BMD and Etest methods demonstrated that voriconazole was very active against all species of *Aspergillus* (98 to 99% susceptible at a MIC of ≤1 μg/ml) and superior to itraconazole (62 to 84% susceptible at a MIC of ≤1 μg/ml). By using the BMD results, ≥90% of *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. versicolor*, *A. nidulans*, and *A. sydowii* isolates were inhibited by ≤1 μg of voriconazole/ml. By comparison, with the exception of *A. flavus* (97% inhibited at a MIC of ≤1 μg/ml), only 48 to 89% of these species were inhibited by ≤1 μg of itraconazole/ml.

As reported by Espinel-Ingroff and Rezusta (10), the agreement between BMD and Etest for both voriconazole and itraconazole was excellent (Table 1). The agreement between BMD and Etest for voriconazole ranged from 75.0% for eight isolates of miscellaneous *Aspergillus* spp. (*A. glaucus*, *A. nidulans*, *A. ustus*, and *A. sydowii*) to 100% for *A. flavus* and was

TABLE 1. MICs for voriconazole and itraconazole determined by the reference BMD and Etest methods and percent agreement between the methods for 376 isolates of *Aspergillus* spp.

Species (no. tested), antifungal agent, and test method	% Susceptible at MIC (μg/ml):								% Agreement[a]
	0.06	0.12	0.25	0.5	1	2	4	8	
A. fumigatus (277)									
Voriconazole									
BMD[b]	0	14	86	97	99	99	100		
Etest	8	53	94	99	99	99	100		98.9
Itraconazole									
BMD	0	0	2	33	88	99	99	99	
Etest	1	1	3	15	64	93	99	99	97.8
A. flavus (32)									
Voriconazole									
BMD	3	6	31	84	100				
Etest	9	13	59	94	100				100.0
Itraconazole									
BMD	0	0	13	72	97	100			
Etest	0	3	13	25	69	94	100		100.0
A. niger (27)									
Voriconazole									
BMD	0	4	19	74	96	100			
Etest	4	22	70	93	100				92.6
Itraconazole									
BMD	0	0	0	15	48	96	100		
Etest	0	0	4	4	4	22	74	89	89.5
A. terreus (19)									
Voriconazole									
BMD	5	11	68	100					
Etest	16	58	68	100					94.7
Itraconazole									
BMD	0	0	21	100					
Etest	16	37	74	89	89	100			89.5
A. versicolor (13)									
Voriconazole									
BMD	8	15	46	62	92	100			
Etest	15	46	69	77	77	85	92	100	92.3
Itraconazole									
BMD	0	8	15	23	54	100			
Etest	0	8	15	38	62	69	77	100	92.3
Aspergillus spp.[c] (8)									
Voriconazole									
BMD	25	38	63	63	63	75	88	88	
Etest	75	75	75	75	88	88	100		75.0
Itraconazole									
BMD	0	0	0	50	50	88	88	88	
Etest	0	0	63	63	75	88	88	88	75.0
Total (376)									
Voriconazole									
BMD	1	13	73	93	98	99	99	99	
Etest	10	48	87	97	99	99	99	100	97.6
Itraconazole									
BMD	0	1	4	38	84	99	99	99	
Etest	1	3	9	20	62	88	97	99	95.8

[a] Percent agreement: percentage of Etest MICs within 2 dilutions of the reference BMD MICs determined following 48-h incubation.
[b] BMD method performed according to NCCLS M38-A (16).
[c] Includes *A. glaucus* (one isolate), *A. nidulans* (three isolates), *A. ustus* (two isolates), and *A. sydowii* (two isolates).

97.6% overall (Table 1). Likewise, for itraconazole, the agreement ranged from 75.0% for *Aspergillus* spp. to 100% for *A. flavus* and was 95.8% overall. When discrepancies between the BMD and Etest MICs were noted, the Etest tended to give lower values with voriconazole and higher values with itraconazole.

The results of this study confirm and extend previous observations with itraconazole (22) and those of Espinel-Ingroff and Rezusta (10) with both itraconazole and voriconazole in testing *Aspergillus* spp. by BMD and Etest. Espinel-Ingroff and Rezusta (10) found that using RPMI agar and 48-h incubation resulted in an overall agreement of 96.3% for voriconazole and 82.2% for itraconazole. The agreement between BMD and Etest for itraconazole improved to 91.8% when the Etest was read after 24-h incubation (10). Previously, an agreement of 100% was demonstrated at both 24 and 48 h for the itraconazole BMD and Etest results when testing *Aspergillus* spp. (22). Due to the faint growth of several isolates after 24 h of incubation in the present study, the 24-h Etest results were not included in our analysis.

Similar to Espinel-Ingroff and Rezusta (10), we found the highest levels of agreement for voriconazole with the two most common species, *A. fumigatus* and *A. flavus.* Whereas Espinel-Ingroff and Rezusta (10) reported only 70% agreement for voriconazole and *A. nidulans*, we found 100% agreement with this species (data not shown).

In summary, we have provided additional documentation of the ability of Etest to generate voriconazole and itraconazole MIC data for *Aspergillus* spp. that are comparable to those obtained by the NCCLS BMD method. RPMI agar with 2% glucose may be used to determine the reference quality MICs of voriconazole and itraconazole, as well as posaconazole (10), when tested against *Aspergillus* spp., as well as *Candida* spp. (15, 22, 24). The value of these data is enhanced by the utilization of a large collection of geographically diverse strains of *Aspergillus*, including nine different species.

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